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| (54) Title: RECOMBINANT STAPHYLOCOCCUS THIOREDOXIN REDUCTASE, AND INHIBITORS THEREOF USEFUL AS ANTIMICROBIAL AGENTS (57) Abstract Isolated and purified <i>Staphylococcus</i> thioredoxin reductases (TrxB) are provided. Polynucleotides encoding the TrxBs, vectors and host cells containing such polynucleotides are also provided. In addition, antibodies reactive with the TrxBs are provided, as are methods of isolating the TrxBs, as well as methods for producing recombinant TrxBs, using TrxBs for screening compounds for TrxB-modulating activity, and detecting <i>Staphylococcus</i> in a test sample. | | |

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RECOMBINANT STAPHYLOCOCCUS THIOREDOXIN REDUCTASE, AND INHIBITORS THEREOF USEFUL AS ANTIMICROBIAL AGENTS

This application claims priority to the provisional application Serial No. 60/076,525 filed on March 2, 1998.

5 Technical Field

This invention relates generally to microbial metabolism and antimicrobial therapeutic agents. In particular, the invention relates to the bacterial enzyme thioredoxin reductase, to compounds that inhibit this enzyme, and to the use of these compounds as antimicrobial agents, particularly for the therapy of infections
10 caused by *Staphylococcus* spp.

Background of the Invention

The thioredoxin system is composed of NADPH, thioredoxin (Trx) and the flavoenzyme thioredoxin reductase (TrxB). Trx reduction by TrxB involves two half-
15 reactions. In the first half-reaction, the FAD prosthetic group of TrxB is reduced by NADPH and electrons are transferred to cysteines present in the active site of TrxB. In the second half-reaction, oxidized Trx is reduced by TrxB. The thioredoxin system serves to transfer reducing equivalents for reductive enzymes such as ribonucleotide reductase, methionine sulfoxide reductase and vitamin K epoxide
20 reductase. It also mediates protein folding and exerts specific redox control of some transcription factors to modulate their binding to DNA.

The thioredoxin system is of particular importance for redox metabolism in some Gram-positive bacteria. In this regard, certain Gram-positive bacteria, such as staphylococci, lack detectable glutathione (GSH) and glutathione reductase (GSR)
25 which together play a key role in maintaining intracellular thiol-disulfide balance. GSH is the predominant thiol produced by aerobic eukaryotes and some Gram-positive bacteria, is believed to protect aerobic organisms from oxygen toxicity, and participates in a multitude of functions. For example, GSH plays a pivotal role in management of oxidative stress and maintenance and regulation of

the redox balance. It acts as a cofactor for peroxide and ribonucleotide reductions, and serves in the conjugation and detoxification of foreign substances. Most organisms contain millimolar intracellular concentrations of GSH which, in concert with GSR and glutathione peroxidase, governs the redox status of the cellular environment. Thus, in microorganisms lacking the glutathione system, such as *Staphylococcus aureus*, the thioredoxin system, which is able to substitute for some of the glutathione-dependent processes, is of utmost importance.

The TrxB component of the thioredoxin system is a FAD-containing enzyme and belongs to a family of pyridine nucleotide-disulfide oxidoreductases. The bacterial enzyme obtained from *Streptomyces clavuligerus*, is a homodimer of 35 kDa subunits and has a native molecular weight of approximately 70 kDa. Aharonowitz *et al.* (1993) *J. Bacteriol.* 175:623-629. Each subunit of TrxB contains NADPH- and FAD- binding domains and includes an oxidoreductase active dithiol in the conserved sequence -CATVC-. Since the cysteine residues of TrxB are relatively inaccessible to the substrate thioredoxin, the enzyme appears to undergo a large conformational change during catalysis.

TrxBs from different mammalian species including calf (Holmgren, A. (1997) *J. Biol. Chem.* 272:4600-4606), rat (Luthman *et al.* (1982) *Biochem.* 21:66628-66633), and human (Arscott, *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:3621-3626), have been purified and biochemically characterized. The rat liver TrxB has been isolated as a 116 kDa homodimer of 58 kDa subunits, and the mass of human placental TrxB estimated to be 160 kDa by gel-filtration chromatography and 130 kDa (two 65 kDa subunits) by sucrose density gradient centrifugation. The size difference between human TrxB and the smaller bacterial TrxBs is primarily due to differences in the dimer-interface domain. The redox-active cysteines of human TrxB are located in the FAD domain with a 4-amino acid bridge linking the two cysteines. The active-site disulfide of bacterial TrxBs, on the other hand, is located within the NADPH domain and a 2-amino acid bridge links the two cysteines. The 3D structure of the human TrxB is likely to be more similar to GSR than to the bacterial TrxB. Thus, primary amino acid sequence alignment of human TrxB with

bacterial TrxB sequences reveals just 23-31% identity, whereas alignment of the human TrxB with GSRs of different sources shows 35-44% identity. Arscott, *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:3621-3626.

Based on the significant differences that exist between the bacterial and the mammalian *trx*B genes, and the enzymes encoded thereby, the bacterial TrxB provides a potential target for the development of novel antibacterial drugs with a high degree of selectivity.

Antimicrobial agents commonly used to combat bacterial infections generally interfere with one or more critical steps in the metabolism of the bacterium, resulting in growth inhibition or death of the microbe. However, pathogenic microorganisms, including staphylococci, are developing resistance, and in many cases multiple resistances, to existing antimicrobial agents. In this regard, *S. aureus* is an opportunistic pathogen of increasing medical concern. It can be aggressively invasive, spreading rapidly through soft tissues, directly invading bones and even entering the bloodstream to produce septic shock and disseminated intravascular coagulation. Infections caused by staphylococci generally fall within one of two categories: those related to toxins produced by the bacterium exclusively, including gastroenteritis, toxic shock syndrome, scalded skin syndrome, and the like; and those related to direct invasion and systemic spread of the organism, including dermal infections, bone and joint infections, staphylococcal pneumonia and empyema, meningitis, cerebritis, endocarditis, bacteremia, septic shock, and the like.

These staphylococcal infections have traditionally been treated with β -lactam antibiotics. However, strains of β -lactam antibiotic-resistant staphylococci (BLARS), such as methicillin-resistant *S. aureus* (MRSA), have developed and become a widespread cause of fatal nosocomial infection. Infections caused by such resistant staphylococci are treated predominantly with "last resort" antibiotics such as vancomycin. Since resistance to these antibiotics would essentially exhaust the current therapeutic arsenal, it is essential that new antibacterial agents be identified.

Summary of the Invention

The inventors herein have identified a bacterial thioredoxin reductase (TrxB) from *Staphylococcus* spp. that catalyzes, in two half-reactions, the specific NADPH-dependent reduction of thioredoxin (Trx), with the concomitant oxidation of NADPH to NADP⁺. In the first half-reaction, the FAD prosthetic group of TrxB is reduced by NADPH and electrons are transferred to cysteines present in the active site of TrxB. In the second half-reaction, oxidized Trx is reduced by TrxB.

The thioredoxin system provides a significant metabolic function in staphylococci and other Gram-positive bacteria that do not produce glutathione (GSH). The thioredoxin system catalyzes a broad range of protein thiol-disulfide exchange reactions, donates hydrogen for ribonucleotide reductase which is an essential enzyme in DNA synthesis, and is involved in redox regulation of numerous enzyme activities.

Staphylococcal TrxB differs significantly in its function from that of the mammalian enzyme in two important ways. First, it operates in an intracellular environment lacking GSH and GSH-dependent reductases. Second, its subunits are appreciably smaller than the mammalian enzyme and the dimeric enzyme possesses a substrate specificity distinct from its mammalian counterpart. Inhibition of *Staphylococcal* TrxB activity may cause depletion of reduced low molecular weight thiols, increase protein thiol oxidation, and interfere with DNA synthesis and radical scavenging. Such compromised cells are more likely to succumb to environmental challenges, such as those posed by the host immune system.

Consequently, bacterial TrxB provides an excellent target for the development of novel antibacterial drugs with a high degree of selectivity.

Such antibacterials act by inhibiting TrxB, thereby incapacitating the target bacterium, with few or no side-effects to the eukaryotic host organism. As a result, inhibitors of TrxB activity are effective antimicrobial agents against *Staphylococcus* and other microorganisms that depend on TrxB for their redox reactions.

Accordingly, in one embodiment, the invention is directed to an isolated *Staphylococcus* TrxB polypeptide.

In another embodiment, the invention is directed to an isolated polynucleotide that encodes a *Staphylococcus* TrxB polypeptide.

In yet other embodiments, the invention is directed to a recombinant vector comprising the polynucleotide molecule, recombinant host cells transformed with the vector, and methods of producing recombinant polypeptides using the transformed cells.

In still a further embodiment, the invention is directed to an oligonucleotide probe capable of specifically hybridizing to a nucleic acid molecule encoding a *Staphylococcus* TrxB. The probe includes about 8 to about 50 contiguous nucleotides of the TrxB-encoding polynucleotide.

In still another embodiment of the invention, antibodies to a *Staphylococcus* TrxB polypeptide are provided.

In another embodiment, the invention is directed to a method of detecting the presence of *Staphylococcus* in a test sample suspected of containing a *Staphylococcus*, comprising:

(a) contacting the test sample with the oligonucleotide probe above, under conditions which allow a *Staphylococcus* polynucleotide, when present in the test sample, to hybridize with the oligonucleotide probe to form a hybrid complex; and

(b) detecting the presence or absence of the hybrid complex as an indication of the presence of a *Staphylococcus* in the test sample.

In yet another embodiment, the invention is directed to a method of detecting the presence of *Staphylococcus* in a test sample suspected of containing *Staphylococcus*, comprising:

(a) contacting the test sample with a TrxB antibody, under conditions which allow a *Staphylococcus* TrxB, when present in the test sample, to bind to the antibody to form an antibody-TrxB complex; and

(b) detecting the presence or absence of the antibody-TrxB complex, as an indication of the presence of *Staphylococcus* in the test sample.

In another embodiment, the invention is directed to a method of detecting a *Staphylococcus* in a test sample suspected of containing a *Staphylococcus*, comprising:

- (a) incubating the test sample with a disulfide-containing substrate in the presence of thioredoxin and NADPH, under conditions that favor reduction of the disulfide-containing substrate, to produce a detectable signal when a *Staphylococcus* thioredoxin reductase is present;
- (b) detecting the presence or absence of the signal; and
- (c) correlating the presence of the signal with the presence of *Staphylococcus* in the test sample.

In yet further embodiments, the invention is directed to diagnostic kits comprising an oligonucleotide probe as above, a TrxB antibody, or a *Staphylococcus* TrxB polypeptide, and instructions for conducting the diagnostic test.

In another embodiment, the invention is directed to a method for identifying a compound that modulates *Staphylococcus* TrxB activity, comprising:

- (a) providing a *Staphylococcus* TrxB polypeptide capable of catalyzing the specific reduction of thioredoxin with the concomitant oxidation of NADPH to NADP⁺;
- (b) contacting a test compound with the TrxB polypeptide in the presence of thioredoxin, NADPH and a disulfide-containing substrate, under conditions that favor reduction of the disulfide-containing substrate;
- (c) monitoring the presence of free sulfhydryl groups formed by the reduction of disulfides of the disulfide-containing substrate, as a measure of TrxB activity,

thereby identifying a compound that modulates *Staphylococcus* TrxB activity.

In yet further embodiments, the invention is directed to compounds identified by this method, compositions comprising the compounds and methods of treating *Staphylococcus* infections in an infected subject, comprising administering effective antibacterial amounts of the compositions

In another embodiment, the invention is directed to a method for isolating a TrxB polypeptide from a *Staphylococcus* cell culture extract, comprising:

(a) performing a protein precipitation step with the cell culture extract to yield a first TrxB mixture;

(b) subjecting the first TrxB mixture to gel-filtration chromatography;

(c) identifying fractions from step (b) with TrxB activity; and

5 (d) performing anion-exchange chromatography on the fractions with TrxB activity to yield a product with a greater concentration of TrxB than the first TrxB mixture.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

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Brief Description of the Figures

Figure 1 depicts the nucleotide sequence (SEQ ID NO:1) of the *S. aureus* *trxB* open reading frame.

Figure 2 shows the deduced amino acid sequence (SEQ ID NO:2) of *S. aureus* TrxB derived from the nucleotide sequence of the open reading frame.

15

Figure 3 is a diagram of vector pAUL-A.

Figure 4 is a diagram of disruption vector pOI7.

Figures 5A and 5B show the method used to construct the gene replacement vector pOI5. Figure 5A shows the construction of plasmid pOI4 used to construct plasmid pOI5, shown in Figure 5B.

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Figure 6 depicts the nucleotide sequence (SEQ ID NO:9) of the *S. epidermidis* *trxB* open reading frame.

Figure 7 shows an alignment of the TrxB enzymes from *S. aureus* (designated TrxB_Sa, SEQ ID NO:2) and *S. epidermidis* (designated TrxB_Se, SEQ ID NO:10). Colons indicate conservative amino acid substitutions and single dots indicate somewhat conservative substitutions.

25

Figure 8 is a flow chart depicting a method for identifying TrxB inhibitors using an insulin turbidity test.

Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology and recombinant DNA technology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989); *DNA Cloning*, Vols. I and II (D.N. Glover ed. 1985); the series, *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); Scopes, *Protein Purification: Principles and Practice* (2nd ed., Springer-Verlag); and *PCR: A Practical Approach* (McPherson *et al.* eds. (1991) IRL Press).

All patents, patent applications and publications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The term "polynucleotide" as used herein means a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, the term includes double- and single-stranded DNA, as well as double- and single-stranded RNA. It also includes modifications, such as by methylation and/or by capping, and unmodified forms of the polynucleotide.

"Polypeptide" and "protein" are used interchangeably herein and indicate a molecular chain of amino acids linked through peptide bonds. The terms do not refer to a specific length of the product. Thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. The terms include post-translation modifications of the polypeptide, for example, glycosylations, acetylations,

phosphorylations, and the like. In addition, protein fragments, analogs, muteins, fusion proteins and the like are included within the meaning of polypeptide.

Thus, by "TrxB polypeptide" is meant a polypeptide, whether isolated, recombinant or synthetic, comprising an amino acid sequence identical to that depicted in Figures 2 (SEQ ID NO:2) or 10 (SEQ ID NO:10), and fragments thereof that include as much of the molecule as necessary for the polypeptide to retain biological activity, e.g., catalytic and/or immunological activity, as well as analogs, mutated or variant proteins, and the like, thereof that retain such activity. Generally, if catalytic activity is required, the polypeptide will include the NADPH- and FAD-binding domains, as well as a redox active site. The NADPH domain is found at residues 146-161 of Figures 2 and 10. Two FAD domains are present, and found at residues 6-21 and 267-277, of Figures 2 and 10. Finally, the redox active site is found at residues 134-137 and includes the amino acid sequence CAVC. Thus, if catalytic activity is desired, the polypeptide can include about amino acid residues 1-277 or more of Figures 2 or 10, or will include the above specified portions, with internal regions deleted, so long as catalytic activity is retained. If immunological activity is desired, e.g., the ability to raise antibodies for use in assays, immunopurification, and the like, the protein will contain one or more epitopes capable of eliciting a humoral antibody response. Normally, an epitope will include between about 3-20 amino acids, generally about 8-10, and preferably about 15-20 or more amino acids, derived from the TrxB molecule.

By sequence "similarity" between two amino acid sequences is meant an exact amino acid to amino acid comparison of two or more polypeptides at appropriate portions of the molecule, where identical amino acids are aligned, and where residues are not identical, aligned based on similar chemical and/or physical properties such as charge or hydrophobicity. "Percent similarity" can be determined between the compared polypeptide sequences using techniques well known in the art.

In general, "identity" refers to an exact nucleotide to nucleotide or amino acid to amino acid correspondence of two or more polynucleotide or polypeptide

sequences, respectively. Techniques for determining nucleic acid and amino acid "sequence identity" are well known in the art and include determining the nucleotide sequence of the mRNA for the gene of interest (usually via a cDNA intermediate) and determining the amino acid sequence encoded thereby, and comparing this to a second amino acid sequence. Programs available in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, WI) for example, the GAP program, are capable of calculating both the identity between two polynucleotides and the identity and similarity between two polypeptide sequences. Other programs for calculating identity or similarity between sequences are known in the art.

Generally, a polypeptide analog of TrxB will have at least about 50% identity, preferably about 60% identity, more preferably about 75-85% identity, and most preferably about 90-95% or more identity, to the reference TrxB sequence. Further, the polypeptide may have at least about 60% similarity, preferably at least about 75% similarity, more preferably about 85% similarity, and most preferably about 95% or more similarity to the reference sequence. Additionally, a polynucleotide variant will display at least about 50% identity, preferably about 60% identity, more preferably about 75-85% identity, and most preferably about 90-95% or more identity, to the reference *trxB* nucleotide sequence.

By the term "degenerate variant" or "structurally conserved mutation" is intended a polynucleotide containing changes in the nucleic acid sequence thereof, such as insertions, deletions or substitutions, that encodes a polypeptide having the same amino acid sequence as the polypeptide encoded by the polynucleotide from which the degenerate variant is derived.

"Recombinant host cells," "host cells," "cells," "cell lines," "cell cultures," and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be, or have been, used as recipients for recombinant vectors or other transfer DNA, immaterial of the method by which the DNA is introduced into the cell or the subsequent disposition of the cell. The terms include the progeny of the original cell which has been transfected.

A "vector" is a replicon in which another polynucleotide segment is attached, such as to bring about the replication and/or expression of the attached segment. The term includes expression vectors, cloning vectors, and the like.

5 The term "control sequence" refers to a polynucleotide sequence which effects the expression of coding sequences to which it is ligated. The nature of such a control sequence differs depending upon the host organism. In prokaryotes, such control sequences generally include a promoter, a ribosomal binding site, and a terminator. In eukaryotes, such control sequences generally include a promoter, a terminator and, in some instances, an enhancer. The term "control sequence" thus
10 is intended to include at a minimum all components necessary for expression, and also may include additional components whose presence is advantageous, for example, leader sequences.

A "coding sequence" is a polynucleotide sequence that is transcribed into mRNA and/or translated into a polypeptide when placed under the control of
15 appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to, mRNA, cDNA, and recombinant polynucleotide sequences. Mutants or analogs may be prepared by the deletion of a portion of the coding sequence, by insertion of a
20 sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. See, e.g., Sambrook *et al.*, *supra*; *DNA Cloning*, Vols. I and II, *supra*; *Nucleic Acid Hybridization*, *supra*.

"Operably linked" refers to a situation wherein the components described are
25 in a relationship permitting them to function in their intended manner. Thus, for example, a control sequence "operably linked" to a coding sequence is ligated in such a manner that expression of the coding sequence is achieved under conditions compatible with the control sequences. A coding sequence may be operably linked to control sequences that direct the transcription of the polynucleotide whereby said
30 polynucleotide is expressed in a host cell.

"Transformation" refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion, or the molecular form of the polynucleotide that is inserted. For example, injection, direct uptake, transduction, and f-mating are included. Furthermore, the insertion of a polynucleotide *per se* and the insertion of a plasmid or vector comprised of the exogenous polynucleotide, are also included. The exogenous polynucleotide may be directly transcribed and translated by the cell, maintained as a nonintegrated vector, for example, a plasmid, or alternatively, may be stably integrated into the host genome.

The term "probe" denotes a defined nucleic acid segment which can be used to identify a specific polynucleotide present in samples bearing the complementary sequence. A probe will generally include about 8 to about 75 contiguous nucleic acids of the reference polynucleotide, generally about 12 to about 50 contiguous nucleic acids, and preferably about 15-18 to about 30 contiguous nucleic acids of the reference sequence.

The term "isolated," when referring to a polynucleotide or a polypeptide, intends that the indicated molecule is present in the substantial absence of other similar biological macromolecules of the same type. The term "isolated" as used herein means that at least 75 wt.%, more preferably at least 85 wt.%, more preferably still at least 95 wt.%, and most preferably at least 98 wt.% of a composition is the isolated polynucleotide or polypeptide. An "isolated polynucleotide" that encodes a particular polypeptide refers to a polynucleotide that is substantially free of other nucleic acid molecules that do not encode the subject polypeptide; however, the molecule may include conservative mutations as defined herein. Thus, an isolated polynucleotide would not be a polynucleotide as it exists on the chromosome.

The term "test sample" refers to a component of an individual's body which is the source of an analyte, such as antibodies or antigens of interest. These test samples include biological samples which can be tested by the methods of the present invention described herein and include human and animal body fluids such

as whole blood, serum, plasma, cerebrospinal fluid, urine, lymph fluids, and various external secretions of the respiratory, intestinal and genitorurinary tracts, tears, saliva, milk, white blood cells, myelomas and the like; biological fluids such as cell culture supernatants; fixed tissue specimens; and fixed cell specimens.

5 The following single-letter amino acid abbreviations are used throughout the text:

| | | | | |
|----|---------------|---|---------------|---|
| | Alanine | A | Arginine | R |
| | Asparagine | N | Aspartic acid | D |
| | Cysteine | C | Glutamine | Q |
| 10 | Glutamic acid | E | Glycine | G |
| | Histidine | H | Isoleucine | I |
| | Leucine | L | Lysine | K |
| | Methionine | M | Phenylalanine | F |
| | Proline | P | Serine | S |
| 15 | Threonine | T | Tryptophan | W |
| | Tyrosine | Y | Valine | V |

B. General Methods

The present invention is based on the identification of novel *Staphylococcal*
 20 TrxB polypeptides, including *S. aureus* TrxB (also termed TrxB_Sa herein) and *S. epidermidis* TrxB (also termed TrxB_Se herein), as well as polynucleotides encoding the TrxBs, and methods of making the TrxBs. The invention includes not only the enzyme but also methods for screening compounds for pharmacological activity using the enzyme, cells expressing the enzyme, antibodies to the enzyme and the
 25 use of polynucleotides and antibodies to diagnose the presence of *Staphylococcus* in a test sample.

In particular, the inventors herein have identified new TrxB enzymes in *S. aureus* and *S. epidermidis*. The native enzyme has a molecular mass (M_r) of approximately 70 kDa, as determined by gel filtration chromatography. The enzyme
 30 exists as a homodimer having a subunit M_r of about 35 kDa, as determined by SDS

polyacrylamide gel electrophoresis. The predicted value of the *Staphylococcal* TrxB subunit molecular mass, based on the primary amino acid sequence is 33.518, and the calculated isoelectric point (PI) is 5.39. The gene encoding the *S. aureus* TrxB enzyme has been cloned and the ORF coding for the TrxB protein is shown in Figure 1. The gene encodes a putative amino acid sequence of 311 amino acids, shown in Figure 2.

Similarly, the gene encoding the *S. epidermidis* TrxB enzyme has been identified and the nucleotide and amino acid sequences are shown in Figures 6 and 7, respectively.

The enzyme can be isolated directly from bacteria as follows. Bacteria are cultured in a suitable culture medium, such as trypticase soy broth (TSB). The bacteria are then removed from the culture medium using standard techniques known in the art, such as by centrifugation or microfiltration or a combination of the two. For example, microfiltration using an appropriate filter will suffice to remove unwanted cellular debris.

Bacteria thus obtained are prepared to release the contents of the cytoplasm. Bacterial cells may be broken using methods and/or reagents known in the art that do not adversely affect the structure and/or the activity of the TrxB, e.g., exposure to freeze-thaw cycles, exposure to an ultrasonic disintegrator, homogenization, bead milling, chemical or enzymatic cell lysis, and the like. In one preferred method, cells are incubated in a buffer containing lysostaphin, a lytic agent for *S. aureus*, and then centrifuged to remove insoluble cellular debris.

The bacterial cell extract thus prepared can be further processed to separate the protein from the cellular debris, and provide an initial stage of purification and volume reduction. For example, the extract obtained from the previous step may be processed by a primary separation procedure such as ultrafiltration, i.e., passage through a filter having an appropriate molecular weight cut-off, to concentrate the sample by reducing the water and salt content. Alternatively, the extract may be precipitated by neutral salts such as ammonium sulphate, organic solvents such as ethanol, or other agents for recovering and purifying the protein. Preferably,

Staphylococcus TrxB is precipitated from the extract by adding ammonium sulfate to the extract to approximately 30% to 50% saturation, preferably 40% saturation. The supernatant of the same is collected by, e.g., centrifugation, and the ammonium sulfate is adjusted to about 70% to 90%, preferably 80% saturation. The treated
5 precipitate thus obtained is collected and used in further purification steps.

A number of protein purification operations may be used to further purify the *Staphylococcus* TrxB including adsorption chromatography, ion-exchange chromatography, hydrophobic interaction chromatography, affinity chromatography, chromatofocussing, gel-filtration, reversed-phase liquid chromatography,
10 phosphocellulose chromatography, hydroxyapatite chromatography or lectin chromatography, any combination of such techniques. Protein refolding steps can be used, as necessary, in completing configuration of the protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

15 For example, the fraction precipitating between 40-80% ammonium sulfate saturation can be applied to a gel-filtration matrix for size separation. Useful matrices for size separation include any of several known in the art such as, without limitation, those made of dextran, dextran/bisacrylamide, polyacrylamide, agarose, cross-linked agarose, agarose/polyacrylamide, and cross-linked polyethers.
20 Particularly preferred matrices are those which allow fractionation of molecules in the size range of about 10^4 to 10^6 , such as Sephacryl S-300, Sepharose 6B, Sepharose CL-6B, Ultragel AcA44, Ultragel AcA34, Ultragel A6, and the like.

Fractions from the size-separation step which exhibit TrxB and Trx activity can be pooled and applied to an ion-exchange column and proteins eluted with a
25 linear salt gradient. Preferred ion-exchangers are anion-exchangers, well known in the art. Particularly preferred herein are relatively weak anion-exchangers, such as those including aminoethyl (AE) or diethylaminoethyl (DEAE) functionalities. Useful matrix materials include but are not limited to, cellulose matrices, such as fibrous, microgranular and beaded matrices; agarose, dextran, polyacrylate, polyvinyl,

polystyrene, silica and polyether matrices; and composites. A representative anion-exchanger for use herein is DEAE-Sepharose.

TrxB activity can be monitored during purification using standard TrxB assays. For example, the presence of TrxB can be assayed spectrophotometrically by monitoring the NADPH- and TrxB disulfide-dependent reduction of a disulfide-containing substrate. One assay method monitors the reduction of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) at 412 nm. See, e.g., Ellman (1959) *Arch. Biochem. Biophys.* 82:70-77. A particularly preferred assay uses insulin as the disulfide-containing substrate. The presence of free sulfhydryl (SH) groups in insulin are measured at 650 nm as an indication of TrxB activity. See, e.g., A. Holmgren and M. Bjornstedt, (1995) *Methods in Enzymology* 252:199-208; and the examples herein. In this reaction, TrxB specifically reduces thioredoxin (T-S₂) to T-(SH)₂ using NADPH. The formed T-(SH)₂ in turn reduces native insulin. Insulin can be replaced by any of several low molecular weight disulfide compounds in the assay, such as cystine, and TrxB activity is monitored spectrophotometrically following the oxidation of NADPH.

Following isolation, purity of fractions showing TrxB activity can be determined using any of several methods such as by SDS-PAGE.

Once purified, the amino acid sequences of the proteins can be determined, e.g., by repetitive cycles of Edman degradation, followed by amino acid analysis by HPLC. Other methods of amino acid sequencing are also known in the art. Based on knowledge of the amino acid sequence, DNA encoding the enzyme can be derived from genomic or cDNA, prepared by synthesis, or by a combination of techniques. The DNA can then be used to express the TrxB, or as a template for the preparation of RNA, using methods well known in the art (see, Sambrook *et al.*, *supra*).

More particularly, DNA encoding the *Staphylococcus* TrxB may be obtained from an appropriate DNA library, e.g., an *S. aureus* or *S. epidermidis* genomic DNA library. DNA libraries may be probed using the procedure described by Grunstein *et al.* (1975) *Proc. Natl. Acad. Sci. USA* 73:3961. Briefly, the DNA to be probed is

immobilized on nitrocellulose filters, denatured and prehybridized with a buffer which contains 0-50% formamide, 0.75 M NaCl, 75 mM Na citrate, 0.02% (w/v) each of bovine serum albumin (BSA), polyvinyl pyrrolidone and Ficoll®, 50 mM Na phosphate (pH 6.5), 0.1% sodium dodecyl sulfate (SDS) and 100 µg/ml carrier denatured DNA. The percentage of formamide in the buffer, as well as the time and temperature conditions of the prehybridization and subsequent hybridization steps, depends on the stringency required. Oligomeric probes which require lower stringency conditions are generally used with low percentages of formamide, lower temperatures, and longer hybridization times. Probes containing more than 30 or 40 nucleotides such as those derived from cDNA or genomic sequences generally employ higher temperatures, for example, about 40°C to 42°C, and a high percentage, for example, 50%, formamide. Following prehybridization, a ³²P-labelled oligonucleotide probe is added to the buffer, and the filters are incubated in this mixture under hybridization conditions. After washing, the treated filters are subjected to autoradiography to show the location of the hybridized probe. DNA in corresponding locations on the original agar plates is used as the source of the desired DNA.

Synthetic oligonucleotides may be prepared using an automated oligonucleotide synthesizer such as that described by Warner (1984) *DNA* 3:401. If desired, the synthetic strands may be labeled with ³²P by treatment with polynucleotide kinase in the presence of ³²P-ATP, using standard conditions for the reaction. DNA sequences including those isolated from genomic or cDNA libraries, may be modified by known methods which include site-directed mutagenesis as described by Zoller (1982) *Nucleic Acids Res.* 10:6487. Briefly, the DNA to be modified is packaged into phage as a single stranded sequence, and converted to a double stranded DNA with DNA polymerase using, as a primer, a synthetic oligonucleotide complementary to the portion of the DNA to be modified, and having the desired modification included in its own sequence. Cultures of the transformed bacteria, which contain replications of each strand of the phage, are plated in agar to obtain plaques. Theoretically, 50% of the new plaques contain phage having the

mutated sequence, and the remaining 50% have the original sequence. Replicates of the plaques are hybridized to labeled synthetic probe at temperatures and conditions suitable for hybridization with the correct strand, but not with the unmodified sequence. The sequences which have been identified by hybridization
5 are recovered and cloned.

Once produced, the DNA may then be incorporated into a cloning or expression vector for replication in a suitable host cell. Vector construction employs methods known in the art. Generally, site-specific DNA cleavage is performed by treating with suitable restriction enzymes under conditions which generally are
10 specified by the manufacturer of these commercially available enzymes. Usually, about 1 microgram (μg) of plasmid or DNA sequence is cleaved by 1-10 units of enzyme in about 20 μl of buffer solution by incubation at 37°C for 1 to 2 hours. After incubation with the restriction enzyme, protein is removed by phenol/chloroform extraction and the DNA recovered by precipitation with ethanol. The cleaved
15 fragments may be separated using polyacrylamide or agarose gel electrophoresis methods, according to methods known by those of skill in the art.

Sticky end cleavage fragments may be blunt ended using *E. coli* DNA polymerase 1 (Klenow) in the presence of the appropriate deoxynucleotide triphosphates (dNTPs) present in the mixture. Treatment with S1 nuclease also may
20 be used, resulting in the hydrolysis of any single stranded DNA portions.

Ligations are performed using standard buffer and temperature conditions using T4 DNA ligase and ATP. Sticky-end ligations require less ATP and less ligase than blunt-end ligations. When vector fragments are used as part of a ligation mixture, the vector fragment often is treated with bacterial alkaline phosphatase
25 (BAP) or calf intestinal alkaline phosphatase to remove the 5'-phosphate and thus prevent religation of the vector. Alternatively, restriction enzyme digestion of unwanted fragments can be used to prevent ligation.

For standard vector constructions, ligation mixtures are transformed into a suitable host, and successful transformants selected by antibiotic resistance or other
30 markers. Plasmids from the transformants can then be prepared according to the

method of Clewell *et al.* (1969) *Proc. Natl. Acad. Sci. USA* 62:1159 usually following chloramphenicol amplification as reported by Clewell *et al.* (1972) *J. Bacteriol.* 110:667. The DNA is isolated and analyzed usually by restriction enzyme analysis and/or sequencing. Sequencing may be by the well-known dideoxy method of Sanger *et al.* (1977) *Proc. Natl. Acad. Sci. USA* 74:5463) as further described by Messing *et al.* (1981) *Nucleic Acid Res.* 9:309, or by the method reported by Maxam *et al.* (1980) *Meth. Enzymol.* 65:499. Problems with band compression, which are sometimes observed in GC rich regions, are overcome by use of T-deazoguanosine according to the method reported by Barr *et al.* (1986) *Biotechniques* 4:428.

Host cells are genetically engineered (transduced, transformed, or transfected) with the vectors of this invention which may be a cloning vector or an expression vector. The vector may be in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the TrxB-encoding polynucleotide. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to those of skill in the art.

Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences when appropriate control sequences that are compatible with the designated host are used. Among prokaryotic hosts, *E. coli* is frequently used. Expression control sequences for prokaryotes include promoters, optionally containing operator portions, and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from the plasmid pBR322 that contains operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, that also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Commonly used prokaryotic control sequences include the β -lactamase (penicillinase), lactose promoter system (Chang *et al.* (1977) *Nature* 198:1056), the tryptophan promoter system (reported by Goeddel *et al.* (1980) *Nucleic Acid Res.* 8:4057) and the lambda-derived P_l promoter and N gene ribosome binding site

(Shimatake *et al.* (1981) *Nature* 292:128) and the hybrid *Tac* promoter (De Boer *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 292:128) derived from sequences of the *trp* and *lac* UV5 promoters. The foregoing systems are particularly compatible with *E. coli*; however, other prokaryotic hosts such as strains of *Bacillus* or *Pseudomonas* may be used if desired, with corresponding control sequences.

Eukaryotic hosts include yeast and mammalian cells in culture systems. *Saccharomyces cerevisiae* and *S. carlsbergensis* are commonly used yeast hosts, and are convenient fungal hosts. Yeast-compatible vectors carry markers that permit selection of successful transformants by conferring protrophy to auxotrophic mutants or resistance to heavy metals on wild-type strains. Yeast compatible vectors may employ the 2-micron origin of replication (Broach *et al.* (1983) *Meth. Enzymol.* 101:307), the combination of CEN3 and ARS1 or other means for assuring replication, such as sequences that will result in incorporation of an appropriate fragment into the host cell genome. Control sequences for yeast vectors are known in the art and include promoters for the synthesis of glycolytic enzymes, including the promoter for 3-phosphoglycerate kinase. See, for example, Hess *et al.* (1968) *J. Adv. Enzyme Reg.* 7:149, Holland *et al.* (1978) *Biochemistry* 17:4900, and Hitzeman (1980) *J. Biol. Chem.* 255:2073. Terminators also may be included, such as those derived from the enolase gene as reported by Holland (1981) *J. Biol. Chem.* 256:1385. It is contemplated that particularly useful control systems are those that comprise the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter or alcohol dehydrogenase (ADH) regulatable promoter, terminators also derived from GAPDH, and, if secretion is desired, leader sequences from yeast alpha factor. In addition, the transcriptional regulatory region and the transcriptional initiation region which are operably linked may be such that they are not naturally associated in the wild-type organism.

Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines which are available from the American Type Culture Collection. These include HeLa cells, Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, and others. Suitable promoters for

mammalian cells also are known in the art and include viral promoters such as that from Simian Virus 40 (SV40), Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV), cytomegalovirus (CMV). Mammalian cells also may require terminator sequences and poly A addition sequences; enhancer sequences which
5 increase expression also may be included, and sequences which cause amplification of the gene also may be desirable. These sequences are known in the art. Vectors suitable for replication in mammalian cells may include viral replicons, or sequences which insure integration of the appropriate sequences encoding the *Staphylococcus* TrxB into the host genome.

10 Other eukaryotic systems are also known, as are methods for introducing polynucleotides into such systems, such as into amphibian cells, using known methods, and insect cells using methods described in Summers and Smith (1987), *Texas Agricultural Experiment Station Bulletin No. 1555*, and the like.

Transformation may be by any known method for introducing polynucleotides
15 into a host cell, including packaging the polynucleotide in a virus and transducing a host cell with the virus, by direct uptake of the polynucleotide by the host cell, and the like. The transformation procedures selected depend upon the host to be transformed. Bacterial transformation by direct uptake generally employs treatment with calcium or rubidium chloride. Cohen (1972) *Proc. Natl. Acad. Sci. USA*
20 69:2110. Yeast transformation by direct uptake may be conducted using the calcium phosphate precipitation method of Graham *et al.* (1978) *Virology* 52:526, or modification thereof.

Expression of active TrxB can be assayed spectrophotometrically, as described above, by monitoring the NADPH- and TrxB disulfide-dependent reduction
25 of a disulfide-containing substrate. Particularly useful substrates include DTNB and insulin. See, e.g., Ellman (1959) *Arch. Biochem. Biophys.* 82:70-77; and A. Holmgren and M. Bjornstedt, (1995) *Methods in Enzymology* 252:199-208. Alternatively, expression of TrxB can be monitored using an ELISA assay and antibodies prepared to the isolated TrxB enzyme. The enzyme is recovered and

purified from recombinant host cell cultures expressing the same by known methods as described above.

The *Staphylococcus* TrxB polypeptide or fragments thereof, may also be synthesized by conventional techniques known in the art, for example, by chemical synthesis such as solid-phase peptide synthesis. In general, these methods employ either solid- or solution- phase synthesis methods, well known in the art. See, e.g., J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, 2nd Ed., Pierce Chemical Co., Rockford, IL (1984) and G. Barany and R. B. Merrifield, *The Peptides: Analysis, Synthesis, Biology*, editors E. Gross and J. Meienhofer, Vol. 2, Academic Press, New York, (1980), pp. 3-254, for solid phase peptide synthesis techniques; and M. Bodansky, *Principles of Peptide Synthesis*, Springer-Verlag, Berlin (1984) and E. Gross and J. Meienhofer, Eds., *The Peptides: Analysis, Synthesis, Biology*, *supra*, Vol. 1, for classical solution synthesis.

Once obtained, the enzyme may be used to identify compounds that modulate *Staphylococcus* TrxB activity. Thus, as described above, enzyme activity and the effects of compounds on enzyme activity can be assayed spectrophotometrically by monitoring the NADPH- and TrxB disulfide-dependent reduction of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) at 412 nm, or by measuring the presence of free SH groups of insulin at 650 nm.

Purification or expression of *Staphylococcus* TrxB, and screening for compounds that inhibit the enzyme activity, provides a method for rapid selection of compounds with enzyme-inhibiting activity.

Compounds that inhibit *Staphylococcus* TrxB are considered potential therapeutic agents for use in treating several disorders caused by staphylococcal bacteria, including without limitation those caused by *S. aureus*, *S. epidermidis*, *S. albus*, *S. hyicus*, *S. hyos*, *S. intermedius*, *S. simulans*, and the like. These bacteria cause infection in man and other nonhuman primates, as well as in domestic animals, such as dogs and cats, and in farm animals, including horses, pigs, sheep, goats and cows. These agents are therefore useful in preventing or retarding

growth and/or reproduction of the infecting microorganism in a wide variety of vertebrate subjects.

Examples of diseases for which TrxB inhibitors are useful therapeutic agents include any of the several infections caused by staphylococci including, but not limited to, gastroenteritis, enterocolitis, toxic shock syndrome, scalded skin syndrome, dermal infections, cellulitis, toxic epidermal necrolysis, ecthyma, necrotizing fasciitis, folliculitis, furuncles, carbuncles, impetigo, bone and joint infections, osteomyelitis, pneumonia and empyema, meningitis, cerebritis, endocarditis, bacteremia, septic shock, septicemia, food poisoning, enteritis, and the like.

The inhibitory compounds of the present invention can be formulated into pharmaceutical compositions in a variety of dosage forms such as, but not limited to, liquid solutions or suspensions, tablets, pills, powders, ointments suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions. The preferred form depends upon the mode of administration and the particular microorganism and disease targeted. The compositions also preferably include pharmaceutically acceptable vehicles, carriers or adjuvants, well known in the art, such as human serum albumin, ion exchangers, alumina, lecithin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, and salts or electrolytes such as protamine sulfate. Suitable vehicles are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. Actual methods of preparing such compositions are known, or will be apparent, to those skilled in the art. See, e.g., *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pennsylvania, 18th edition, 1990.

The above compositions can be administered using conventional modes of delivery including, but not limited to, intramuscular, intravenous, intraperitoneal, oral, intralymphatic, or subcutaneous administration. Local administration to a tissue in question, or to a site of infection, e.g., direct injection into an infected joint, will also find use with the present invention.

Therapeutically effective doses will be easily determined by one of skill in the art and will depend on the severity and course of the disease, the patient's health and response to treatment, and the judgment of the treating physician.

Furthermore, the *Staphylococcus* TrxB polypeptide can be used to prepare
5 polyclonal or monoclonal antibodies using techniques that are well known in the art. If polyclonal antibodies are desired, a selected mammal, (e.g., mouse, rabbit, goat, horse, etc.) is immunized with a TrxB polypeptide and serum from the immunized animal is collected and treated according to known procedures. See, e.g., Jurgens
10 *et al.* (1985) *J. Chrom.* 348:363-370. If serum containing polyclonal antibodies is used, the polyclonal antibodies can be purified by immunoaffinity chromatography, using known procedures.

Monoclonal antibodies to the TrxB polypeptides can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies using hybridoma technology is well known. Immortal antibody-producing
15 cell lines can be created by cell fusion, as well as by other techniques, such as direct transformation of B-lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier *et al.*, *Hybridoma Techniques* (1980); Hammerling *et al.*, *Monoclonal Antibodies and T-cell Hybridomas* (1981); Kennett *et al.*, *Monoclonal Antibodies* (1980); U.S. Patent Nos. 4,341,761; 4,399,121,
20 4,427,783; 4,444,887; 4,452,570; 4,466,917; 4,472,500, 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against the TrxB polypeptides can be screened for various properties; e.g., for isotype, epitope, affinity, etc. Monoclonal antibodies are useful in purification, using immunoaffinity techniques, of the TrxB polypeptides. Monoclonal and polyclonal antibodies that display specificity and
25 selectivity for the enzyme can be labeled with a detectable moiety, e.g., a fluorescent moiety, and used in *in vitro*, or *in situ* immunofluorescent assays, or the like. The antibodies can be used to identify *Staphylococcus* for immunodiagnostic purposes.

In addition, DNA encoding the *Staphylococcus* TrxBs, or RNA derived
30 therefrom, can be used to design oligonucleotide probes for *Staphylococcus* present

in a host organism. As used herein, the term "probe" refers to a structure comprised of a polynucleotide, as defined above, which contains a nucleic acid sequence complementary to a nucleic acid sequence present in a target polynucleotide. The polynucleotide regions of probes may be composed of DNA, and/or RNA, and/or
5 synthetic nucleotide analogs such as morpholino compounds and peptide nucleic acid ("PNA") analogs. Such probes may be used in *in vitro* or *in situ* hybridization assays, or the like, and are useful, for example, for the diagnosis of microbial infections.

Using a determined portion of the isolated TrxB-encoding polynucleotide,
10 oligomers of approximately eight or more nucleotides can be prepared, either by excision or synthetically, which hybridize with the TrxB-encoding polynucleotide. Such oligomers are useful, for example, for detecting the presence of bacteria in diseased individuals. The natural or derived probes for *trxB* polynucleotides are a length that allows the detection of unique sequences by hybridization. While 6-8
15 nucleotides may be a workable length, sequences of 10-12 nucleotides are preferred, and those of about 18-20 nucleotides, or more, most preferred. These probes can be prepared using routine, standard methods including automated oligonucleotide synthetic methods.

When the oligonucleotide probes are to be used as diagnostic reagents, the
20 test sample to be analyzed, such as blood or serum, may be treated such as to extract a nucleic acid fraction thereof. The resulting nucleic acid from the sample may be subjected to gel electrophoresis or other size separation techniques, or the nucleic acid sample may be dot-blotted without size separation. The sample is then exposed to an oligonucleotide probe that has been detectably labeled. Suitable
25 labels and methods for attaching labels to probes are known in the art, and include but are not limited to radioactive labels incorporated by nick translation or kinasing, biotin, fluorescent and chemiluminescent probes, enzymes which catalyze the production of a detectable product such as horseradish peroxidase, alkaline phosphatase, β -galactosidase, and the like. The nucleic acids extracted from the

sample are then treated with the labeled probe under conditions of suitable hybridization stringency.

The stringency of hybridization is determined by a number of factors during the washing procedure, including temperature, ionic strength, length of time and concentration of formamide. Sambrook *et al.*, *supra*. Hybridization can be carried out by a number of techniques. Amplification of the sample nucleic acid, if required, can be performed, for example, by ligase chain reaction (LCR), polymerase chain reaction (PCR), Q-beta replicase, NASBA, or other techniques well known in the art. The amplified nucleic acids then may be detected using a hybridization assay such as those known in the art.

TrxB, antibodies thereto, as well as polynucleotides encoding TrxB or portions thereof, can be provided in diagnostic kits. For example, oligomer probes capable of specifically hybridizing to a polynucleotide encoding a TrxB can be packaged in diagnostic kits which include the probe nucleic acid sequence which may be labelled. Alternatively, the probe may be provided unlabelled and the ingredients for labelling can be included with the kit. The kit also may contain other suitably packaged reagents and materials needed or desirable for the particular hybridization protocol, for example, standards as well as instructions for performing the assay.

In addition, kits can include reagents for detecting of the presence and/or amount of *Staphylococcus* TrxB in a test sample, as well as for detecting the presence of *Staphylococcus*. Such reagents can comprise, e.g., an antibody capable of specifically binding to the TrxB polypeptide.

Furthermore, kits containing a *Staphylococcus* TrxB polypeptide in a suitable container are provided for screening compounds for TrxB-modulating activity or for screening test samples for the presence of a TrxB antibody. It is contemplated that reagents employed in the above kits can be provided in one or more containers such as vials or bottles, with each container containing a separate reagent such as a monoclonal antibody, or a cocktail of monoclonal antibodies, or a polypeptide (either recombinant or synthetic) employed in the assay. Other components such as

buffers, controls, and the like, known to those of ordinary skill in art, may be included in such test kits. The kits will also include instructions for the use thereof.

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not
5 intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

10 C. Experimental

Example 1

Identification of a Thioredoxin Reductase from *S. aureus*

In order to identify the enzyme responsible for the reduction of oxidized thioredoxin (Trx), *S. aureus* extracts were analyzed for thioredoxin reductase (TrxB)
15 activity on oxidized thioredoxin in a NADPH-dependent reaction specific for the reduction of insulin as the protein disulfide substrate. 10 ml of an overnight culture of *S. aureus* RN8325-4 (obtained from Richard Novick at New York University School of Medicine), grown at 37°C in tryptic soy broth (TSB) medium (Difco Laboratories, Detroit, MI), was used to inoculate 0.5 L fresh sterilized TSB medium
20 and the culture was grown for 4 hours. Cells were harvested by centrifugation (8000 x g, 15 min), resuspended in 50 mM Tris HCl buffer (20 ml) containing 1 mM EDTA and disrupted by sonication. The homogenized suspension was cleared from insoluble cellular debris by centrifugation and saved. The supernatant was brought to a final concentration of 1% streptomycin sulfate, stirred for 30 min and the nucleic
25 acid precipitate removed by centrifugation. Ammonium sulfate was added to the clear liquid and the fraction that precipitated between 40-80% ammonium sulfate saturation was collected by centrifugation, dissolved in a minimal volume of buffer and chromatographed on a AcA44 gel-filtration size column.

Fractions exhibiting TrxB activity were separated from those containing the
30 Trx activity and served as the high and low molecular weight protein components.

respectively, in the thioredoxin assay. High molecular weight protein fractions were added to a reaction mixture containing an excess of Trx, NADPH and insulin, and the formation of free sulfhydryl (SH) groups in insulin was detected by titration with 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), which forms a yellow product (TNB, absorbance maximum at 412 nm) on interaction with free thiols. The reduction of the insulin disulfides is strictly dependent on the presence of each of reaction components (NADPH, TrxB, Trx). DTNB may be used in place of insulin as the substrate for the Trx-driven reaction, but is less specific. See, e.g., Ellman (1959) *Arch. Biochem. Biophys.* 82:70-77.

A typical assay (Table 1) consisted of a 40 μ l fraction of the high molecular weight component from the AcA44 gel-filtration column to which was added 40 μ l of a solution containing HEPES buffer pH 7.6, 0.26 M; EDTA 0.01 M; NADPH 2.0 mM; insulin, 1.03 mM. The reaction was initiated by adding a 20 μ l fraction of the low molecular weight Trx component eluted from the same gel-filtration column and the mixture was incubated for 20 min at 37°C. The reaction was stopped with 500 μ l of a solution containing 0.4 mg DTNB/ml and 6 M Guanidine HCl in 0.2 M Tris HCl, pH 8.0, and the absorbance A_{412} read. Table 1 shows the ability of the *S. aureus* thioredoxin system to reduce insulin disulfides in the presence of NADPH.

| Table 1 Identification of thioredoxin reductase activity in <i>Staphylococcus aureus</i> | | | | | |
|---|-------|------------------|------|---------|-----------|
| Reaction/ Components | NADPH | Trx ^a | TrxB | Insulin | A_{412} |
| Trx | + | - | + | + | 0.493 |
| NADPH | - | + | + | + | 0.150 |
| Complete | + | + | + | + | 0.816 |

^aThe A_{412} reading observed when Trx is omitted in the assay, reflects the presence of some Trx and other thiols in the partially purified TrxB preparation employed.

Example 2

Purification and Characterization of a Thioredoxin Reductase from *S. aureus*

TrxB was purified from cell extracts of *S. aureus* by following the NADPH-
5 dependent reduction of DTNB. An overnight culture of *S. aureus* strain RN8325-4
grown in TSB (100 ml) at 37°C was used as inoculum (5%) for each of two 2L flasks
containing TSB (0.5L). Cells were shaken (200 rpm) for 4 hrs at 37°C before being
harvested by centrifugation (Sorvall GSA rotor, 5000 rpm, 15 min). All subsequent
handling of the sample prior to chromatography was carried out at 4°C. The cell
10 pellet was resuspended in a minimum of TE buffer (50 mM Tris HCl, pH 8.0, 1 mM
EDTA) containing phenylmethylsulphonyl fluoride (PMSF) (1 mM) and lysostaphin
(0.5 mg), incubated at 4°C, stirred for 30 min, disrupted further by sonication and
then centrifuged (15,000 x g, 20 min) to remove insoluble cellular debris.
Streptomycin was added to a final concentration of 1%, stirred for 30 min at 4°C and
15 the nucleic acid precipitate removed by centrifugation. The clear supernatant was
brought to 40% saturation with ammonium sulfate, stirred to 15 min, and centrifuged
(15,000 x g, 10 min). The resulting supernatant was adjusted to 80% saturation with
ammonium sulfate and the precipitate collected by centrifugation. The fraction
precipitating between 40-80% ammonium sulfate saturation was dissolved in a small
20 volume of buffer and applied to an AcA44 gel-filtration column for size separation
chromatography.

Fractions containing the TrxB activity were easily separated from those
containing the Trx activity. Fractions exhibiting TrxB and Trx activity were pooled,
dialyzed and applied to a DEAE-Sepharose ion exchange column. The column was
25 washed with the loading buffer (TE) and eluted with a linear gradient of NaCl (0.0-
0.5 M) in TE buffer. Fractions containing the TrxB activity were collected and used
for further reactions. The purity of fractions showing TrxB activity was determined by
SDS-PAGE. The molecular weight of the monomer was determined to be
approximately 35 kDa using SDS-PAGE.

Example 3Cloning of the Gene Encoding *S. aureus* TrxB

The gene encoding *S. aureus* TrxB (*trxB*_Sa) was isolated and sequenced as described below. Generally, the gene was identified by PCR using degenerate primers, shown in Table 2A, based on the conserved N-terminal and C-terminal sequences, shown in Table 2B, of known bacterial thioredoxin reductases. See, e.g., Russell *et al.* (1988) *J. Biol. Chem.* 263:9015-9019, for the sequence of *E. coli* TrxB; Cohen *et al.* (1993) *J. Bacteriol.* 175:5159-5167, for the sequence of *S. clavuligerus* TrxB; Cohen *et al.* (1994) *J. Bacteriol.* 176:973-984, for the sequence of *Penicillium chrysogenum* TrxB; Dai *et al.* (1996) *J. Mol. Biol.* 264:1044-1057, for the sequence of *Arabidopsis thaliana* TrxB; Lubbers *et al.* (1993) *Eur. J. Biochem.* 217:791-798, for the sequence of *Eubacterium acidominophilum* TrxB; and Chae *et al.* (1994) *J. Biol. Chem.* 269:27670-27678, for the sequence of *Saccharomyces cerevisiae* TrxB. The DNA fragment generated by PCR was labeled and used as a probe in the isolation of a 2.8 kB *Xba*I fragment from *S. aureus* genomic DNA that carried the *trxB* gene. The sequence of the open reading frame and the deduced amino acid sequence are shown in Figure 1 (SEQ ID NO:1) and Figure 2 (SEQ ID NO:2), respectively.

| Table 2A Degenerate Oligodeoxynucleotide Primers Used in the PCR Amplification of an Internal Region of the Gene Encoding TrxB | |
|--|--|
| Oligomer | Sequence ^a |
| P1 | 5'-ACIACIGAIGTIGA(AG)AA(CT)TT(CT)CC(AGCT)GG-3' |
| (N-terminal region) | (SEQ ID NO:3) |
| M6 | 5'-ACGTCICCCIGCIGC(AG)AA(AGCT)AC(AGCT)CC-3' |
| (C-terminal region) | (SEQ ID NO:4) |

^aI denotes inosine.

| Table 2B' | |
|--|-------------------------|
| Peptide Regions Specified by Degenerate Oligonucleotide Primers Used in PCR Amplification of an Internal Segment of the Gene Encoding TrxB | |
| Region | Sequence |
| N-terminal peptide | TEEVENFPG (SEQ ID NO:5) |
| C-terminal peptide | GIFAAGD (SEQ ID NO:6) |

A. Isolation of a Large Internal DNA Fragment of the *trxB* Gene

Degenerate oligodeoxynucleotides which were designed to encode the N-terminal and C-terminal *trxB* sequences were used as primers for PCR of *S. aureus* genomic DNA. The PCR reaction contained the following:

5 100 ng genomic DNA, 50 µm each of

5'-ACIACIGAIGTIGA(AG)AA(CT)TT(CT)CC(AGCT)GG-3'
(SEQ ID NO:3) and

5'-ACGTCICCCIGCIGC(AG)AA(AGCT)AC(AGCT)CC-3'

(SEQ ID NO:4); MgCl₂ (1.5 mM); bovine serum albumin (BSA) (5 µg);
10 deoxynucleotide triphosphates (dNTPs) (0.2 mM each); and 1 x PCR buffer
(Fermentas, MBI). The reaction mixture was incubated at 94°C (1 min), 55°C (2
min) and 72°C (2 min) for 30 cycles. The resulting 700 bp PCR product was cloned
directly using an AT cloning kit (Promega) and sequenced using universal "forward"
and "reverse" primers which are homologous to the flanking region of the multiple
15 cloning site within the plasmid pGEMT (the AT cloning vector (Promega)). The
cloning vector including the 700 bp PCR product was termed pOI1.

B. Cloning and Sequencing of the Gene Encoding TrxB

The fragment from above was labeled with digoxigenin and used to probe
20 Southern blots of *S. aureus* genomic DNA digested with various restriction enzymes.
A single 2.8 kb *Xba*I fragment that hybridized to the probe under stringent conditions
(50% formamide, 42°C) was subcloned into plasmid pUC18 to form pOI2 and
sequenced. Initial sequencing primers were designed to prime within the sequence
of the PCR fragment described above and to sequence into the flanking regions.

New primers were designed within the new sequences and the nucleotide sequence of the entire gene was thus determined stepwise. All of the sequences were confirmed by sequencing both the coding and non-coding strands.

5 C. Sequence Analysis of *S. aureus* *trxB*

The sequence of the *S. aureus* *trxB* gene and the deduced amino acid sequence are shown in Figure 1 (SEQ ID NO:1) and Figure 2 (SEQ ID NO:2), respectively. Using the CLUSTALW Program (Thompson *et al.* (1994) *Nucl. Acid Res.* 22:4673-4680), the *S. aureus* TrxB amino acid sequence was compared to
10 other bacterial TrxBs. All parameters used were the standard default parameters recommended by CLUSTALW (see, web site <http://www2.ebi.ac.uk/clustalw>). Sequence identities found were *Bacillus subtilis* (72%), *Listeria monocytogenes* (65%), *Clostridium litorale* (53%), *Mycobacterium leprae* (45.5%), *Streptomyces coelicolor* (43.7%), *Escherichia coli* (41.9%), *Mycoplasma genitalis* (35%).

15

D. Heterologous Overexpression of *S. aureus* *trxB* in *E. coli*

The open reading frame encoding *S. aureus* TrxB is amplified by the PCR reaction using the N-terminal primer 5'-GGGAATTCCATATGACTGAAATAGATTTTGAT-3' (SEQ ID NO:7) and the C-terminal PCR primer 5'-CCCAAGCTTTTAAGCTTGATCGTTTAA-3' (SEQ ID NO:8).
20 The resulting fragment is digested with *Nde*I and *Hind*III, purified and ligated with vector pET14b (Novagen, Inc. Madison WI) that is digested with the same two enzymes and purified similarly to produce plasmid pTRSA. An overnight culture (10 mL) of *E. coli* BL21 (DE3) cells harboring pTRSA is washed twice in 10 ml of TB
25 medium and used as an inoculum for 1 liter of the same medium containing ampicillin (400 mg/mL). The resulting culture is incubated at 37°C until it reaches mid-stationary phase ($A_{600} \approx 1.2$), induced to express recombinant TrxB (rTrxB) by the addition of IPTG (to 1 mM) and then incubated for an additional 3 hours at 37°C. The cells are harvested and the recombinant enzyme is purified as described in
30 Example 2 for native TrxB, except that lysozyme (2 mg/mL) is used in place of

lysostaphin to assist in disrupting the cells. The purity of the resulting recombinant enzyme is measured by SDS-PAGE and staining with Coomassie brilliant blue.

E. Construction of Gene Replacement Shuttle Vector

5 Plasmid pAUL-A (Chakraborty *et al.* (1992) *J. Bacteriol.* 174:568-574; Figure 3), used for *trxB* inactivation in *S. aureus* and *S. epidermidis*, contains a temperature sensitive origin of replication for *Staphylococcus* such that, when the temperature is raised to 42°C, the plasmid cannot replicate. Additionally, the plasmid contains the origin of replication of pBR322 and is therefore able to propagate in both *E. coli* and
10 in the above bacteria. If the inactivation plasmid carries a segment of the host chromosome, recombination between the homologous segments will result in the integration of the plasmid. At 42°C, the integrated plasmid can be stably maintained employing selection for the genetic marker, erythromycin resistance, carried on the plasmid sequence. If the homologous chromosomal fragment represents a segment
15 internal to a gene, a single recombinational crossover of the plasmid into the chromosome will cause disruption of the gene and result in the inactivation of the corresponding polypeptide. If the homologous chromosomal fragment contains a deletion internal to a gene, a single recombinational crossover of the plasmid into the chromosome generates a tandem arrangement of wild-type and mutant genes
20 separated by the plasmid sequence; a second recombination event results in loss of one of the copies and the intervening plasmid sequence which, if of the wild-type copy, leads to gene replacement and inactivation of the corresponding polypeptide.

F. Disruption of Chromosomal *S. aureus trxB* Gene

25 The disruption plasmid, pOI7 (Figure 4), was made as follows. The PCR 700 bp internal *trxB* fragment was excised from plasmid pOI1 by digestion with *Bam*HI and *Eco*II within the multiple cloning site, and inserted into pAUL-A to form pOI7. *S. aureus* strain RN4220 was transformed with pOI7 by electroporation, as described by Schenk, S. and Laddaga, A. (1992) *Microbiol. Lett.* 94:133-138, and
30 transformants grown at 30°C or 42°C (the non-permissive temperature) on plates

containing erythromycin. Transformants containing a mutant *trxB* gene due to disruption of the chromosomal gene were selected for directly at 42°C. From some 2000 transformants tested in this way, four grew up at 42°C but only one could be further propagated at this temperature. PCR analysis revealed that in this case, and in other cases like it, no evidence was found for stable integration of the disruption vector at the chromosomal *trxB* locus. This supports the view that disruption of *trxB* is a lethal event. Control experiments using the pAUL-A plasmid containing a 800 bp internal segment of the *S. aureus glnA* gene, showed that stable integrants of the plasmid readily occurred at the *glnA* chromosomal locus.

G. Replacement of Chromosomal *S. aureus trxB* Gene

The replacement plasmid was made as follows (see Figures 5A and 5B). The 3.1 kb *PvuII-HindIII tetM* (tetracycline resistance) cassette of pRN6680 (Nesin, *et al.* (1990) *Antimicro. Agents. Chemother.* 34:2273-2276) was blunt end ligated to the *NruI/Eco721* large fragment of plasmid pOI2 to form pOI4 (Figure 5A). Plasmid pOI2 is pUC18 containing the 2.8 kb *trxB XbaI* DNA fragment, as described above. Plasmid pOI4 lacks a large internal segment of the *trxB* gene. The 5.2 kb *Asp718-SalI* fragment of pOI4 was inserted into pAUL-A and the resulting plasmid, pOI5 (Figure 5B) was used to transform by electroporation *S. aureus* RN4220. Transformants were grown at 42°C in the presence of erythromycin and tetracycline. The DNAs of six randomly chosen transformants were analyzed by PCR and it was found in each case that the plasmid had integrated into the chromosome by a single crossover event in the DNA flanking region upstream of the *trxB* gene.

Phage Ø11 transduction was performed to generate the desired gene replacement, as described by Novick, R.P. *Meth. Enzymology* 204:587-636. The phage was grown on one of the above integrants at 42°C in the presence of tetracycline and erythromycin and the lysate used to transduce *S. aureus* RN4220. Transductants, in which the wild-type copy of *trxB* had been replaced with the mutant copy due to a phage mediated double crossover recombination event, were screened for at 42°C on plates containing tetracycline but lacking erythromycin.

None of the 750 transductants scored in this way were found to have lost the plasmid erythromycin marker. PCR analysis showed that the transductants contained both the intact and wild-type *trxB* copies. Alternatively, one of the above integrant strains was propagated for 100 generations (in the presence of tetracycline and absence of erythromycin) to enable spontaneous elimination of either the wild-type or mutant *trxB* copies. None of the 750 clones tested were found to have lost the erythromycin marker. These results indicate that loss of the wild-type *trxB* gene is a lethal event and are consistent with the gene disruption experiments described above.

Example 4

Identification of Genes Encoding Thioredoxin Reductase in *Staphylococcus epidermidis*

The polypeptide sequence of TrxB from *S. aureus* shown in Figure 2 (and designated hereinafter of TrxB_Sa) was used to search for genes having similar sequences (and thus theoretically the same functional activity as *S. aureus* TrxB) in a variety of microorganisms. The database search tool BLAST (Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-410) was employed to search the PathoSeq™ database, version 3.0 (Incyte Pharmaceuticals, Palo Alto, CA). Matches were found in the genomic sequence of *Staphylococcus epidermidis* O-47 (available from Incyte) in the contiguous sequence (contig) designated SEP1c0392. The open reading frame encoding the entire TrxB in the contig was identified and the corresponding polypeptide product deduced. The gene for TrxB in *S. epidermidis* (designated *trxB_Se*) is shown in Figure 6 (SEQ ID NO:9). TrxB_Sa and TrxB_Se were compared by aligning the full-length amino acid sequences using the GAP program. This program considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. A gap creation penalty and gap extension penalty is provided in units of matched bases. The program then creates a gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is used, the program also tracks, for each gap inserted, the length of the gap times the

gap extension penalty. The GAP program uses the alignment method of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453.

The alignment of TrxB_{Se} and TrxB_{Sa} is shown in Figure 7. The sequences show about 90.4% identity and about 93.6% similarity (e.g., conservative amino acid substitutions). The gap weight was 12, length weight 4, quality 1470, ratio 4.727, average match 2.912, average mismatch -2.003, and gaps 0.

Example 5

Cloning of the *S. epidermidis* *trxB* Gene and Overexpression in *E. coli*

The open reading frame encoding the polypeptide TrxB_{Se} is amplified by PCR from genomic DNA of *S. epidermidis* O-47 using the N-terminal primer 5'-GGGAATTCCATATGACTGAAGTAGATTTTGAT-3' (SEQ ID NO:7) and the C-terminal reverse primer 5'-CCCAAGCTTTTAATTATCTTTTAATTCTGT-3' (SEQ ID NO:8). The resulting amplified fragment is digested with the enzymes *Nde*I and *Hind*III and ligated with the vector pET22B(+) (Novagen, Madison WI) digested with the same enzymes. The resulting plasmid, designated pTRSE, is introduced in *E. coli* BL21 (DE3) essentially as in Example 3D. The *E. coli* strain carrying pTRSE is grown and the TrxB_{Se} enzyme is isolated essentially as described in Example 3D and analyzed as in Example 1.

Example 6

Inactivation of *trxB* *Se*

A 750 bp segment internal to *trxB*_{Se}, corresponding to nt 11471-12221, lacking N-terminal and C-terminal sequences coding for essential *trxB* nucleotide binding domains, is amplified by PCR employing the primers 5'-GGGAATTCGTATATGCATCTCGTGCCAAT-3' (SEQ ID NO:7) which contains an *Eco*RI site near the 5' end and 5'-CCCAATTGTACCTCGTACTTTAGTACTCA-3' (SEQ ID NO:8) which contains a *Hind*III site near the 5' end. The amplified fragment is digested with the enzymes *Eco*RI and *Hind*III, and ligated into the vector pAUL-A which is digested with the

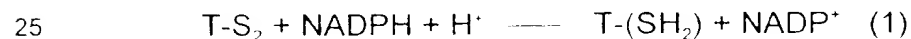
same enzymes. The resulting plasmid, designated pTRSE-int, is introduced into *E. coli*. The *E. coli* strain carrying pTRSE-int is grown and harvested and the plasmid pTRSE-int isolated. The plasmid is electroporated into *S. epidermidis* following the procedures described in Augustin, J. and Goetz, F. (1990) *FEMS Microbiol. Lett.* 68:203-208, employing selection for erythromycin resistance. A culture of *S. epidermidis*/pTRSE-int is grown to mid-log phase in the presence of erythromycin then placed on TSA plates containing erythromycin. To verify that *trxB*_{Se} is an essential gene for growth, the plates are incubated at 42°C and the surviving colonies analyzed for integration of the pTRSE-int plasmid at the chromosomal *trxB* locus due to a single recombinational crossover event. Inability to isolate cells containing a disrupted *trxB* gene indicates that *trxB*_{Se} is an essential gene for growth.

Example 7

15 High Throughput Assay to Identify Inhibitors Of a Microbial Thioredoxin Reductase

An insulin assay (modified from A. Holmgren and M. Bjornstedt, (1995) *Methods in Enzymology* 252:199-208) is used as a sensitive method to monitor the redox-activity of thioredoxin (T) in enabling the NADPH-dependent reduction of insulin disulfides in the presence of thioredoxin reductase (TrxB). In this reaction, TrxB specifically reduces T-S₂ to T-(SH)₂ using NADPH [reaction (1)]. The formed T-(SH)₂ is a powerful reductant of native insulin [reaction (2)].

TrxB



Spontaneous



30 The amount of reduced insulin is determined spectrophotometrically at 412 nm after denaturation with 6M guanidine-HCl in the presence of DTNB step 1 in

scheme. The reduction of insulin in a reaction that is T-dependent and TrxB-independent is tested spectrophotometrically at 650 nm as an increase in turbidity due to the precipitation of the free (reduced) insulin chain B, step 2 in scheme.

A. Step I:

5 One hundred μ l of a reaction mixture containing 0.3 mM insulin, 100 mM HEPES pH 7.5, 3 mM EDTA, 0.5 NADPH, and 1-20 μ g of *S. aureus* TrxB is added to the wells of a series of 96 well microtiter plates. Thioredoxin is omitted from well 1. Wells 3 through 94 contain one of the following: a few μ g of the test compound singly, either as a dry powder or in 1-5 μ l of 50 mM Tris-HCl, pH 7.8, 50 mM NaCl
10 buffer; a few μ g each of a mixture of the test compounds either as a dry powder or in a few μ l of 50 mM Tris-HCl, pH 7.8, 50 mM NaCl buffer, for uncharacterized mixtures of compounds derived from bacteria, fungi, plants, marine organisms and present in a few μ l of water, buffer, or DMSO. In the first two cases, the amount of compound employed is adjusted so that after all the additions are made the concentration of
15 each compound is in the range of 1-10 μ M. Wells 95 and 96 do not contain any compound. To well 1 and to wells 3-96 is added 10 μ l of *S. aureus* TrxB solution containing approximately 300 pmoles in order to start the reaction. Water is added before the addition of TrxB to adjust the volume of each well to 150 μ l. The plate is incubated at 37°C for 20 min. The reaction is terminated by the addition of 150 μ l of
20 0.4 mg/ml DTNB/6 M guanidine hydrochloride in 0.2 M Tris-HCl, pH 8.0 and the absorbance at 412 nm is measured. All compounds that show inhibition of the DTNB reaction are further tested in step II.

B. Step II:

25 (Modified) from A. Holmgren (1979) *J. Biol. Chem.* 254:9627-9632)

Two hundred and fifty μ l of the assay mixture containing a freshly prepared solution of bovine insulin, 1 mg/ml of 0.1 M potassium phosphate pH 7.0, 2 mM EDTA, is added to all the wells of a series of 96 well microtiter plates. Thioredoxin solution, 10 μ l, is added to wells 3-96 to reach 3-8 μ M concentration. Wells 3
30 through 94 also contain one of the following samples that exhibited inhibition of the DTNB reaction in step I, a few μ g of single compounds either as a dry powder or in

1-5 μ l of 50 mM Tris-HCl, pH 7.8, 50 mM NaCl buffer, a few μ g of a mixture of compounds either as a dry powder or in a few μ l of 50 mM Tris-HCl, pH 7.8, 50 mM NaCl buffer; uncharacterized mixtures of compounds derived from bacteria, fungi, plants, marine organisms in a few μ l of water, buffer, or DMSO. In the first two cases, the amount of compound employed is adjusted so that after all the additions are made, the concentration of each is in the range of 1-10 μ M. Wells 95 and 96 do not contain any compound or extract. The reaction is started by the addition of 10 μ l dithiothreitol (DTT) to wells 2-95 to reach a final concentration of 0.3-0.5 mM. The plate is thoroughly agitated and placed in a plate reader and the absorbance at 650 nm is determined. The absorbance is read at intervals of 0.5 min for 80 min. In a typical reaction in the control well 2, containing only DTT and insulin, no precipitation is observed until after about 70 min. In the presence of T and DTT, precipitation appears after 10-15 min.

In order to evaluate whether a compound is a potential TrxB inhibitor, the compound should exhibit the properties depicted in the flow chart shown as Figure 8.

Thus, an isolated *S. aureus* TrxB polypeptide, DNA coding therefor and methods of using the same to identify inhibitors of TrxB, are provided. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

WE CLAIM:

1. An isolated *Staphylococcus* thioredoxin reductase (TrxB) polypeptide.
2. The TrxB polypeptide of claim 1 comprising an amino acid sequence selected from the group consisting of (a) the amino acid sequence of SEQ ID NO:2, (b) the amino acid sequence of SEQ ID NO:10, and (3) an amino acid sequence having 75% identity to either (a) or (b).
3. An isolated polynucleotide encoding the *Staphylococcus* TrxB of Claim 1.
4. An isolated polynucleotide encoding the *Staphylococcus* TrxB of Claim 2.
5. A recombinant vector comprising the polynucleotide of Claim 3 operably linked to control sequences that direct the transcription of the polynucleotide whereby said polynucleotide is expressed in a host cell.
6. A recombinant vector comprising the polynucleotide of Claim 4 operably linked to control sequences that direct the transcription of the polynucleotide whereby said polynucleotide is expressed in a host cell.
7. A host cell comprising the vector of Claim 5.
8. A host cell comprising the vector of Claim 6.

9. A method for producing a *Staphylococcus* TrxB polypeptide comprising:

culturing the host cell of Claim 7 under conditions that allow the production of the TrxB polypeptide; and

5 recovering the TrxB polypeptide.

10. A method for producing a *Staphylococcus* TrxB polypeptide comprising:

10 culturing the host cell of Claim 8 under conditions that allow the production of the TrxB polypeptide; and

recovering the TrxB polypeptide.

11. An oligonucleotide probe capable of specifically hybridizing to a nucleic acid molecule encoding a *Staphylococcus* TrxB, said probe having about 8 to about 50 contiguous nucleotides of the polynucleotide of Claim 3.

12. An oligonucleotide probe capable of specifically hybridizing to a nucleic acid molecule encoding a *Staphylococcus* TrxB, said probe having about 8 to about 50 contiguous nucleotides of the polynucleotide of Claim 4.

13. An antibody reactive with a *Staphylococcus* thioredoxin reductase (TrxB).

14. The antibody of Claim 13, wherein said antibody is a monoclonal antibody.

15. The antibody of Claim 13, wherein said antibody is a polyclonal antibody.

16. A method of detecting the presence of *Staphylococcus* in a test sample suspected of containing *Staphylococcus*, comprising:

(a) contacting the test sample with the oligonucleotide probe of claim 11, under conditions which allow a *Staphylococcus* polynucleotide, when
5 present in the test sample, to hybridize with said oligonucleotide probe to form a hybrid complex; and

(b) detecting the presence or absence of said hybrid complex, as an indication of the presence of *Staphylococcus* in the test sample.

17. A method of detecting the presence of *Staphylococcus* in a test sample suspected of containing *Staphylococcus*, comprising:

(a) contacting the test sample with the oligonucleotide probe of claim 12, under conditions which allow a *Staphylococcus* polynucleotide, when
5 present in the test sample, to hybridize with said oligonucleotide probe to form a hybrid complex; and

(b) detecting the presence or absence of said hybrid complex as an indication of the presence of a *Staphylococcus* in the test sample.

18. A method of detecting the presence of *Staphylococcus* in a test sample suspected of containing *Staphylococcus*, comprising:

(a) contacting the test sample with the antibody of claim 13, under conditions which allow a *Staphylococcus* TrxB, when present in the test sample, to
5 bind to said antibody to form an antibody-TrxB complex; and

(b) detecting the presence or absence of said antibody-TrxB complex, as an indication of the presence of *Staphylococcus* in the test sample.

19. A method of detecting *Staphylococcus* in a test sample suspected of containing *Staphylococcus*, comprising:

- (a) incubating the test sample with a disulfide-containing substrate in the presence of thioredoxin and NADPH, under conditions that favor reduction of the disulfide-containing substrate, to produce a detectable signal when a *Staphylococcus* thioredoxin reductase is present;
- (b) detecting the presence or absence of the signal; and
- (c) correlating the presence of the signal with the presence of *Staphylococcus* in the test sample.

20. A diagnostic test kit comprising:

- (a) a *Staphylococcus* TrxB polypeptide according to Claim 1; and
- (b) instructions for conducting the diagnostic test.

21. A diagnostic test kit comprising:

- (a) an oligonucleotide probe according to Claim 11; and
- (b) instructions for conducting the diagnostic test.

22. A diagnostic test kit comprising:

- (a) an antibody according to Claim 13; and
- (b) instructions for conducting the diagnostic test.

23. A method for identifying a compound that modulates *Staphylococcus* thioredoxin reductase (TrxB) activity, comprising:

(a) providing a *Staphylococcus* TrxB polypeptide capable of catalyzing the specific reduction of thioredoxin with the concomitant oxidation of NADPH to NADP⁺;

(b) contacting a test compound with the TrxB polypeptide in the presence of thioredoxin, NADPH and a disulfide-containing substrate, under conditions that favor reduction of the disulfide-containing substrate;

(c) monitoring the presence of free sulfhydryl groups formed by the reduction of disulfides of the disulfide-containing substrate, as a measure of TrxB activity,

thereby identifying a compound that modulates *Staphylococcus* TrxB activity.

24. The method of Claim 23, wherein the disulfide-containing substrate is insulin.

25. The method of Claim 23, wherein the disulfide-containing substrate is 5,5'-dithio-*bis*-2-nitrobenzoic acid.

26. A compound identified by the method of Claim 23.

27. A composition comprising the compound of Claim 26 and a pharmaceutically acceptable excipient.

28. A method of treating a *Staphylococcus* infection in an infected subject, comprising administering to said subject an effective antibacterial amount of the composition of Claim 27.

29. The method of Claim 28, wherein the infection is a *Staphylococcus aureus* infection.

30. A method for isolating a thioredoxin reductase (TrxB) polypeptide from a *Staphylococcus* cell culture extract, comprising:

(a) performing a protein precipitation step with the cell culture extract to yield a first TrxB mixture;

5 (b) subjecting the first TrxB mixture to gel-filtration chromatography;

(c) identifying fractions from step (b) with TrxB activity; and

(d) performing anion-exchange chromatography on the fractions with TrxB activity to yield a product with a greater concentration of TrxB than the first TrxB mixture.

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| | | | | | |
|------------|------------|------------|------------|------------|-----|
| ATGACTGAAA | TAGATTTTGA | TATAGCAATT | ATCGGTGCAG | GTCCAGCTGG | 50 |
| TATGACTGCT | GCAGTATACG | CATCACGTGC | TAATTTAAAA | ACAGTTATGA | 100 |
| TTGAAAGAGG | TATTCAGGC | GGTCAAATGG | CTAATACAGA | AGAAGTAGAG | 150 |
| AACTTCCCTG | GTTTCGAAAT | GATTACAGGT | CCAGATTTAT | CTACAAAAAT | 200 |
| GTTTGAACAC | GCTAAAAAGT | TTGGTGCAGT | TTATCAATAT | GGAGATATTA | 250 |
| AATCTGTAGA | AGATAAAGGC | GAATATAAAG | TGATTAAGTT | TGGTAACAAA | 300 |
| GAATTAACAG | CTAAAGCCGT | CATTATTGCT | ACAGGTGCAG | GATACAAGAA | 350 |
| AATTGGTGTT | CCGGGTGAAC | AAGAACTTGG | TGGACGCGGT | GTAAGTTATT | 400 |
| GTGCAGTATG | TGATGGTGCA | TTCTTTAAAA | ATAAACGCCT | ATTCGTTATC | 450 |
| GGTGGTGGTG | ACTCAGCAGT | AGAAGAGGGA | ACATTCTTAA | CTAAATTTGC | 500 |
| TGACAAAGTA | ACAATCGTTC | ACCGTCGTGA | TGAGTTACGT | GCACAACGTA | 550 |
| TTTTACAAGA | TAGAGCATTG | AAAAATGATA | AAATAGACTT | TATTTGGAGC | 600 |
| CATACTTTGA | AATCAATTAA | TGAAAAAGAC | GGCAAAGTGG | GTTCTGTGAC | 650 |
| ATTAACGTCT | ACAAAAGATG | GTTCAGAAGA | AACACACGAG | GCTGATGGTG | 700 |
| TATTCATCTA | TATTGGTATG | AAACCATTA | CAGCACCATT | TAAAGACTTA | 750 |
| GGTATTACAA | ATGATGTTGG | TTATATTGTG | ACAAAAGATG | ATATGACAAC | 800 |
| ATCAGTACCA | GGTATTTTTG | CAGCAGGAGA | TGTTCCGGAC | AAAGGTTTAC | 850 |
| GCCAAATTGT | CACTGCTACT | GGCGATGGTA | GTATTGCAGC | ACAAAGTACG | 900 |
| AGCGGATATA | TTGAACATTT | AAACGATCAA | GCTTAA | | 936 |

FIG.1

| | | | | | |
|------------|------------|------------|------------|------------|-----|
| MTEIDFDIAI | IGAGPAGMTA | AVYASRANLK | TVMIERGIPG | GQMANTEEVE | 50 |
| NFPGFEMITG | PDLSTKMFH | AKKFGAVYQY | GDIKSVEDKG | EYKVINFGNK | 100 |
| ELTAKAVIIA | TGAGYKKIGV | PGEQELGGRG | VSYCAVCDGA | FFKNKRLFVI | 150 |
| GGGDSAVEEG | TFLTKFADKV | TIVHRRDELR | AQRILQDRAF | KNDKIDFIWS | 200 |
| HTLKSINEKD | GKVGSVTLTS | TKDGSEETHE | ADGVFIYIGM | KPLTAPFKDL | 250 |
| GITNDVGYIV | TKDDMTTSVP | GIFAAGDVRD | KGLRQIVTAT | GDGSIAAQST | 300 |
| SGYIEHLNDQ | A | | | | 311 |

FIG.2

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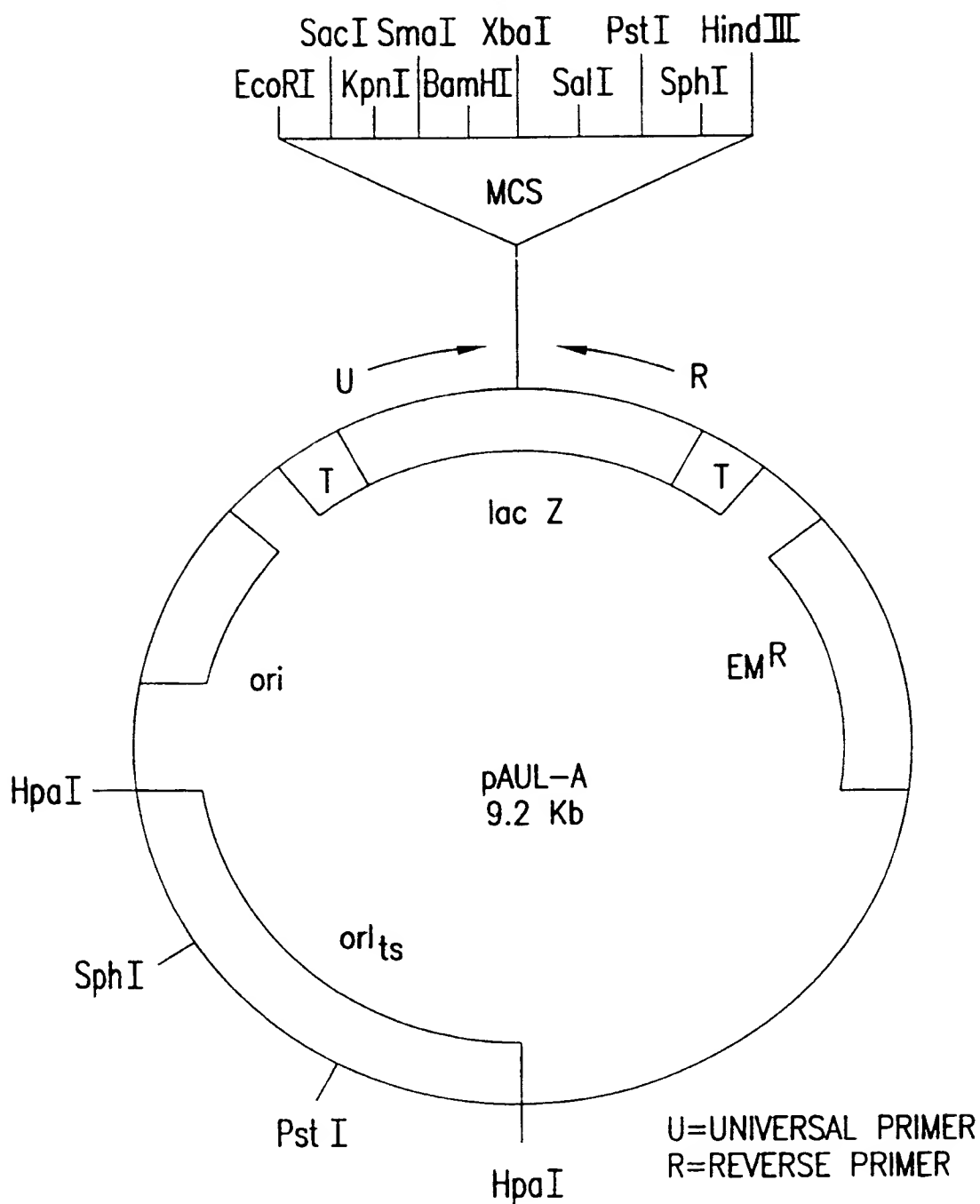


FIG.3

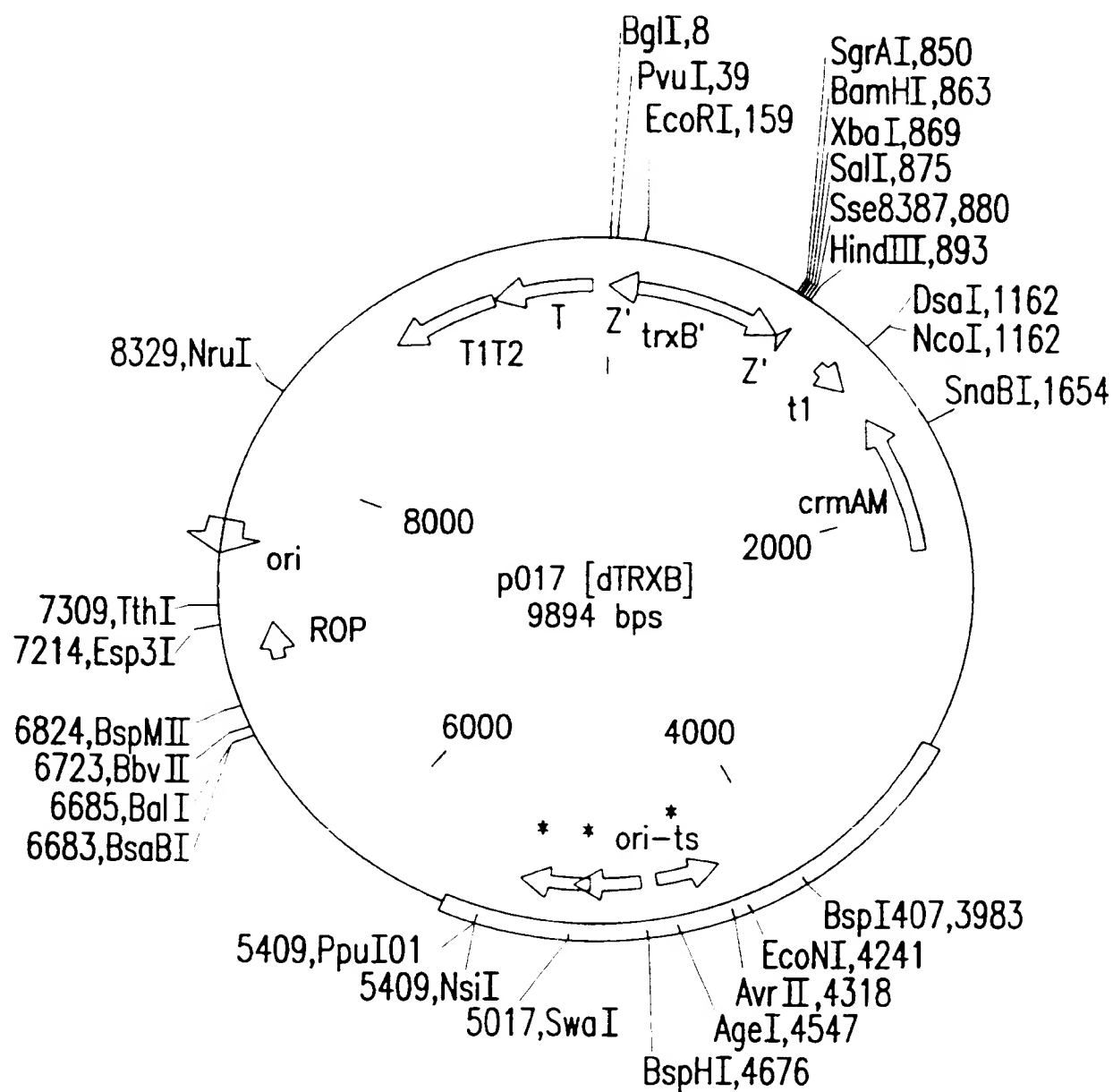


FIG. 4

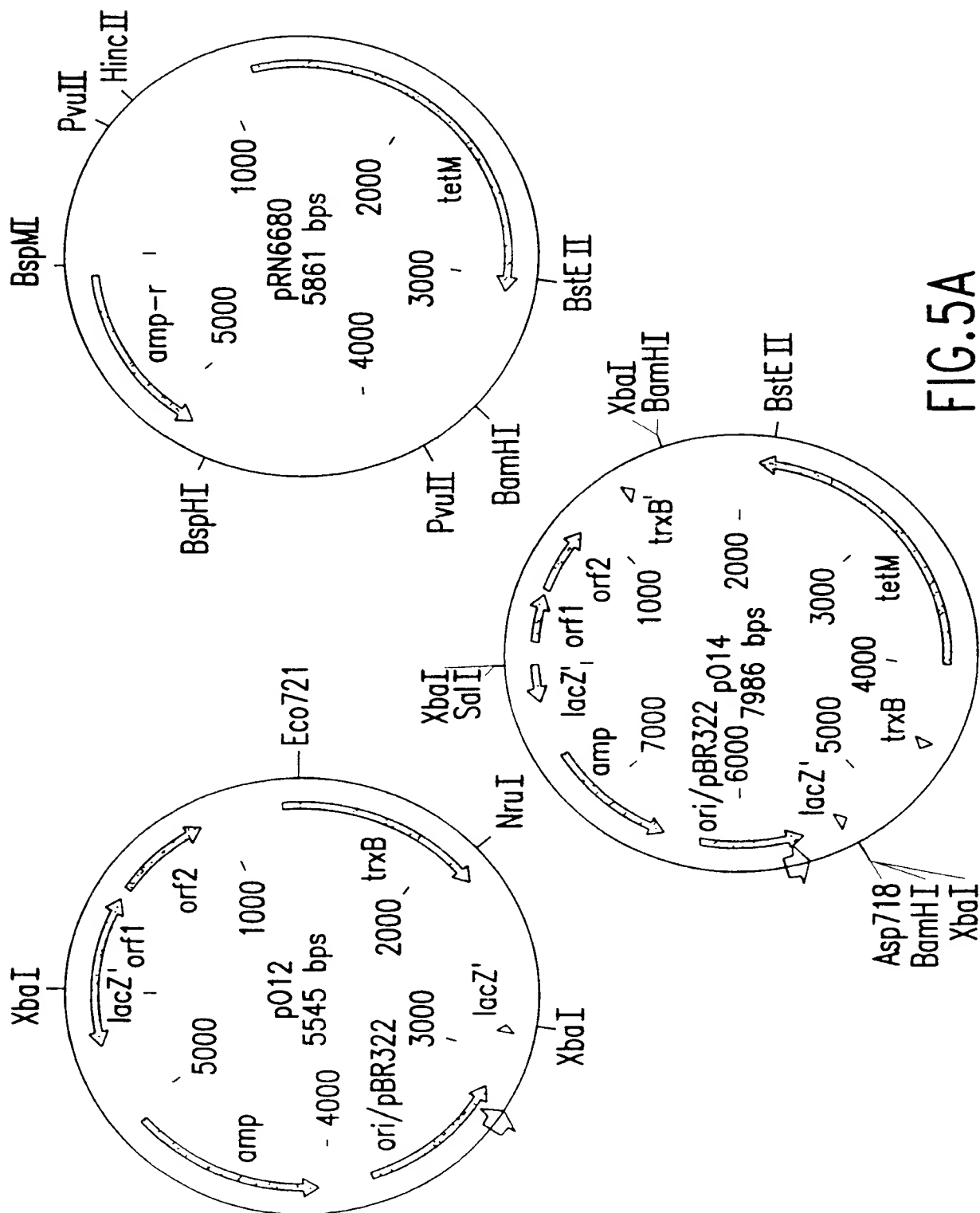


FIG. 5A

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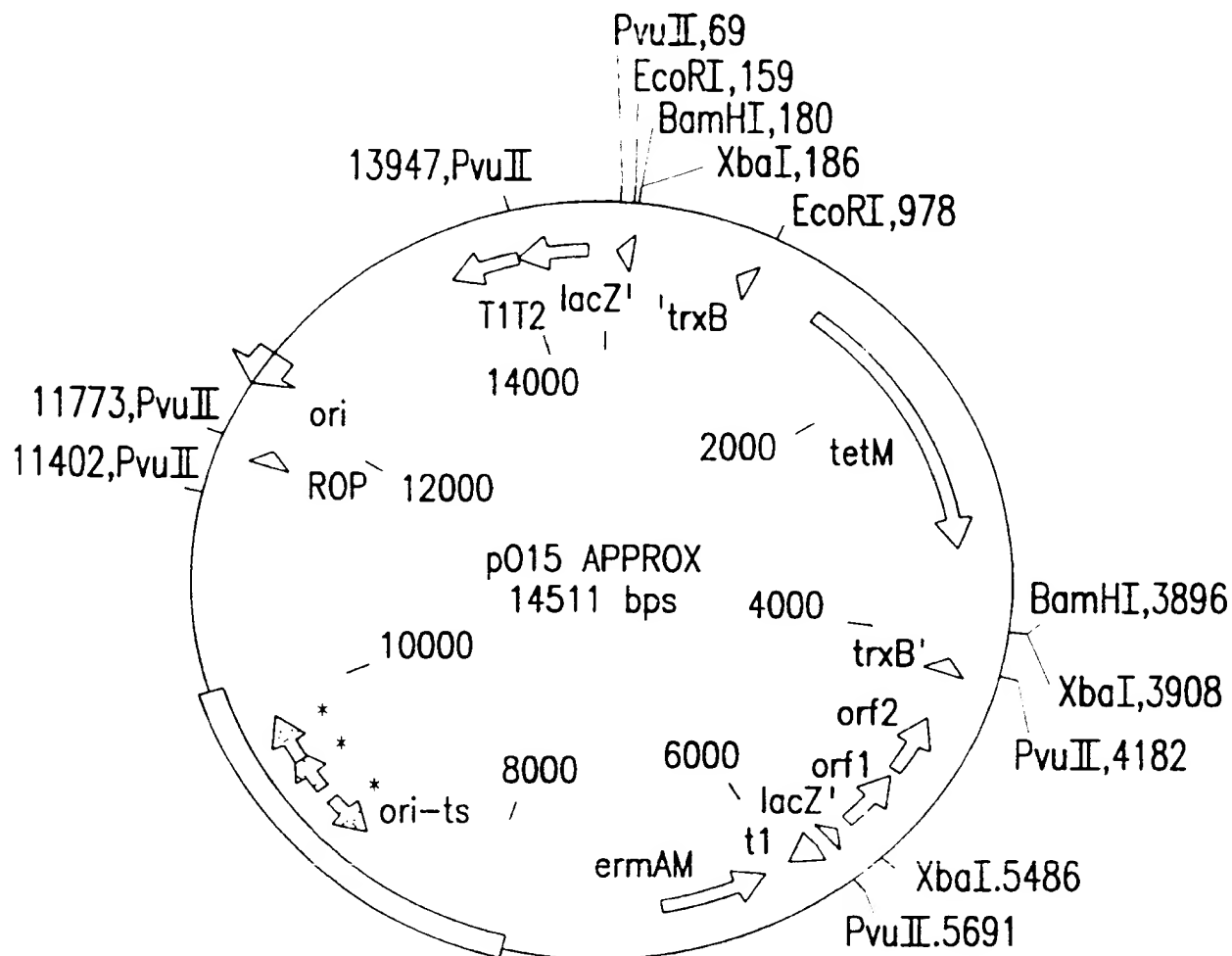


FIG.5B

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1 ATGACTGAAG TAGATTTTGA TGTAGCAATA ATCGGTGCAG GTCCTGCCGG
51 TATGACAGCA GCAGTATATG CATCTCGTGC CAATTTAAAA ACTGTCATGA
101 TTGAACGCCG TATGCCAGGC GGTCAAATGG CAAACACTGA AGAAGTAGAG
151 AATTTTCCAG GATTGAGAT GATCACAGGT CCTGACTTAT CTAATAAAAT
201 GTTGAACAT GCTAAAAAAT TTGGTGCGGA ATACCAATAT GGCGATATTA
251 AATCTGTTGA AGATAAAGGC GACTATAAAG TTATCAATTT AGGGAATAAA
301 GAGATAACAG CACATGCAGT TATTATCTCA ACTGGAGCAG AGTATAAAAA
351 GATTGGCGTT CCTGGTGAAC AAGAATTAGG AGGACGTGGA GTAAGTTATT
401 GTGCGGTTTG TGATGGAGCA TTCTTTAAAA ATAAACGTCT TTTCGTAATT
451 GGCGGCCGAG ATTCAGCGGT AGAAGAAGGT ACTTTCTTAA CTAAATTTGC
501 AGATAAAGTA ACGATTGTTT ACCGTAGAGA TGAATTACGT GCACAAAACA
551 TCTTGCAAGA ACGTGCCTTC AAAAATGATA AAGTTGACTT TATTTGGAGT
601 CATACACTTA AAACAATTAA TGAAAAAGAT GGTAAAGTTG GTTCAGTTAC
651 ACTTGAATCA ACTAAAGATG GTGCTGAACA GACTTATGAT GCCGACGGTG
701 TATTCATTTA TATTGGAATG AAACCACTCA CAGCACCATT TAAAAATCTT
751 GGTATTACAA ATGACGCGGG ATACATTGTC ACACAAGATG ACATGAGTAC
801 TAAAGTACGA GGTATTTTTG CTGCAGGTGA CGTTCGTGAT AAAGGGTTAC
851 GTCAAATTGT TACTGCTACA GGAGACGGTA GTATTGCGGC TCAAAGTGCA
901 GCTGATTATA TTACAGAATT AAAAGATAAT TAA
STOP

FIG.6

SUBSTITUTE SHEET (RULE 26)

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| | | |
|---------|--|-----|
| TrxB_So | MTEIDFDIAIIGAGPAGMTAAVYASRANLKTVMIERGIPGGQMANTEEEVE | 50 |
| | : : : | |
| TrxB_Se | MTEVDFDVAIIGAGPAGMTAAVYASRANLKTVMIERGMPGGQMANTEEEVE | 50 |
| TrxB_So | NFPGFEMITGPDLS TKMFEHAKKFGAVYQYGD IKSVEDKGEYKVINFGNK | 100 |
| | : : : | |
| TrxB_Se | NFPGFEMITGPDLS TKMFEHAKKFGAEYQYGD IKSVEDKGDYKVINLGNK | 100 |
| TrxB_So | ELTAKAVIIATGAEYKKIGVPGEQELGGRGVSYCAVCDGAFFKNKRLFVI | 150 |
| | : : : : | |
| TrxB_Se | EITAHAVIISTGAEYKKIGVPGEQELGGRGVSYCAVCDGAFFKNKRLFVI | 150 |
| TrxB_So | GGGDSAVEEGTFLTKFADKVTIVHRRDELRAQRILQDRAFKNDKIDFIWS | 200 |
| | : : : | |
| TrxB_Se | GGGDSAVEEGTFLTKFADKVTIVHRRDELRAQNILQERAFKNDKVDFIWS | 200 |
| TrxB_So | HTLKSINEKDGVGSVTLTSTKDGSEETHEADGVFIYIGMKPLTAPFKDL | 250 |
| | : : : : : : : | |
| TrxB_Se | HTLKTINEKDGVGSVTLESTKDGAEQTYDADGVFIYIGMKPLTAPFKNL | 250 |
| TrxB_So | GITNDVGYIVTKDDMTTSVPGIFAAGDVRDKGLRQIVTATGDGSIAAQSA | 300 |
| | : : : | |
| TrxB_Se | GITNDAGYIVTQDDMSTKVRGIFAAGDVRDKGLRQIVTATGDGSIAAQSA | 300 |
| TrxB_So | AEYIEHLNDQA | 311 |
| | : | |
| TrxB_Se | ADYITELKDN* | 311 |

FIG.7

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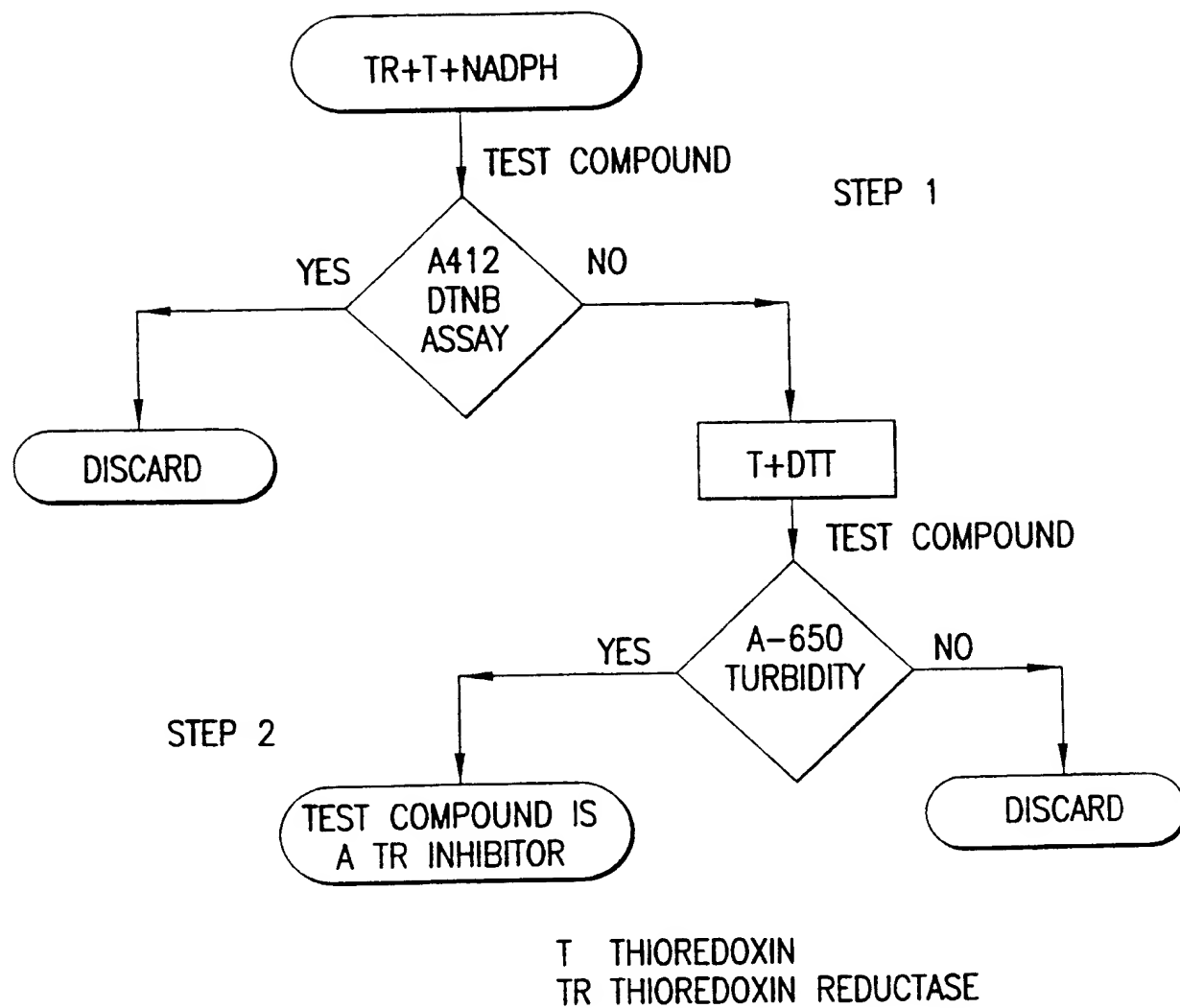


FIG.8

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/04512

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/53 C12N9/02 C12Q1/68 C07K16/40 G01N33/53
C12Q1/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12Q C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No |
|------------|---|----------------------|
| X | EP 0 786 519 A (HUMAN GENOME SCIENCES INC (US); KUNSCH CHOI BARASH DILLON FANNON ROSEN) 30 July 1997 (1997-07-30) | 1-22 |
| Y | abstract page 16, line 14 - page 21, line 42 Seq.ID:3906 page 64 Seq.ID:16 page 101 Seq.ID:3069 page 118 Seq.ID:3733 page 128 Seq.ID:16 nt.8798-9733 page 258 - page 265 Seq.ID:3069 page 2430 - page 2431 Seq.ID:3733 page 2664 | 23-29 |
| | - / - - | |

☒ Further documents are listed in the continuation of box C☒ Patent family members are listed in annex

* Special categories of cited documents

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Z document member of the same patent family

Date of the actual completion of the international search

19 August 1999

Date of mailing of the international search report

25.08.99

Name and mailing address of the ISA

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Fax: (+31-70) 340 3016

Authorized officer

Macchia, G

INTERNATIONAL SEARCH REPORT

International Application No.

T/US 99/04512

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|----------|--|-----------------------|
| Y | Seq.ID:3906 page 2740 - page 2741 page 3267 - page 3269; claims --- HOLMGREN A. AND BJÖRNSTED M.: "Thioredoxin and Thioredoxin Reductase" METHODS IN ENZYMOLOGY, vol. 252, no. part B, 1995, pages 199-208, XP002112640 cited in the application the whole document --- | 23-29 |
| X | WO 97 23628 A (UNIV. BRITISH COLUMBIA (CA); DELCARDAYRE STEPHEN B.; DAVIES JULIAN E.) 3 July 1997 (1997-07-03) page 1 - page 5 page 27 page 39 - page 43; claims --- | 1,30 23-29 |
| A | Sequence Database EMBL ID 054079 Accession number 054079 1 June 1998 100% identity with Seq.ID:2 90% identity with Seq.ID:10 XP002112443 the whole document --- | 1,2 |
| P,X | Sequence Database EMBL ID SAAJ3781 Accession number AJ223781 10 Merch 1998 99% identity with Seq.ID:1 81% identity with Seq.ID:9 XP002112444 the whole document ----- | 3,4 |

INTERNATIONAL SEARCH REPORT

International application No
PCT/US 99/04512

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

- 1 ☒ Claims Nos. .
because they relate to subject matter not required to be searched by this Authority, namely
Remark: Although claims 28, 29 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
- 2 ☐ Claims Nos.
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically
- 3 ☐ Claims Nos.
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6 4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- 1 ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims
- 2 ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee
- 3 ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos
- 4 ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims, it is covered by claims Nos

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest
- ☐ No protest accompanied the payment of additional search fees

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

T/US 99/04512

| Patent document cited in search report | | Publication date | Patent family member(s) | | Publication date |
|---|---|---------------------|----------------------------|-----------|---------------------|
| EP 0786519 | A | 30-07-1997 | CA | 2194411 A | 06-07-1997 |
| | | | JP | 9322781 A | 16-12-1997 |
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| WO 9723628 | A | 03-07-1997 | CA | 2241105 A | 03-07-1997 |
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